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Chlamydia trachomatis Transformants Show a Significant Reduction in Rates of Invasion upon Removal of Key Tarp Domains

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CHLAMYDIA TRACHOMATIS TRANSFORMANTS SHOW A SIGNIFICANT REDUCTION IN RATES OF INVASION UPON REMOVAL OF KEY TARP DOMAINS

by

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ABSTRACT

Chlamydia trachomatis is an obligate, intracellular bacterium which is known to cause multiple human infections including nongonococcal urethritis (serovars D-K), lymphogranuloma venereum (serovars L1, L2, L3) and trachoma (serovars A-C). The infectious form of the bacterium, called the elementary body (EB), harbors a type III secreted effector known as Tarp (translocated actin recruiting phosphoprotein) which is a candidate virulence factor and is hypothesized to play a role in *C. trachomatis'* ability to invade and grow within epithelial cells in a human host. *C. trachomatis* L2 Tarp harbors five unique protein domains which include the Phosphorylation Domain, the Proline Rich Domain, the Actin Binding Domain, and two F-Actin Binding Domains. Tarp has been biochemically characterized *in vitro*, but it has yet to be characterized *in vivo* due to a lack of genetic tools in *C. trachomatis*. Through the recent generation of a chlamydial transformation system, we have created transformants which express epitope tagged wild type or mutant Tarp effectors. In this thesis, *C. trachomatis* transformants expressing Tarp lacking one of the five biochemically defined protein domains were used to examine both bacterial invasion and bacterial development within mammalian host cells. Our results demonstrate that those EBs which harbor mutant Tarp missing either its Phosphorylation Domain or its Actin Binding Domain were less capable of host cell invasion. However, these transformants, once internalized, were capable of normal development when compared to wild type *C. trachomatis or C. trachomatis* harboring an epitope tagged wild type Tarp effector. These results suggest that transformant expressed Tarp lacking the Phosphorylation Domain or Actin Binding Domain may be acting as a dominant-negative effector protein. Ultimately, these results support the hypothesis that Tarp is a virulence factor for *Chlamydia trachomatis*. Furthermore, this data indicates that through the manipulation of the Tarp effector, *C. trachomatis* pathogenesis may be attenuated.

I dedicate this dissertation to both my mother and father, Patricia and Christopher L. Parrett. Without their love and support throughout my life, I would not be half the scientist I am today. Thank you for instilling in me the sense of hard work and dedication required to excel in this field. In addition, I would like to dedicate this work to the late Dr. Bill Safranek who supported me throughout both my undergraduate and the first year of my graduate career. Thank you for all of your helpful advice and for always showing me the brighter side of life.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ACRONYMS

ABD	Actin Binding Domain
CDC	Centers for Disease Control and Prevention
COMC	Chlamydial Outer Membrane Complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
EB	Elementary Body
FAB	F-Actin Binding Domain
F-actin	Filamentous Actin
FBS	Fetal Bovine Serum
G-Actin	Globular Actin
GAG	Glycosaminoglycan
GFP	Green Fluorescent Protein
HS	Heparin Sulfate
IFU	Inclusion Forming Unit
kDa	KiloDaltons
MOMP	Major Outer Membrane Protein
NIH	National Institutes of Health
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-Kinase
PMP	Polymorphic Outer Membrane Protein
PRD	Proline Rich Domain
RB	Reticulate Body
RNP	RNA-Protein Complex
SEM	Standard Error of the Mean
SHC1	Src Homology 2 Domain Containing Protein 1
STD	Sexually Transmitted Disease
Tarp	Translocated Actin Recruiting Phosphoprotein
TepP	Translocated Early Phosphoprotein
WHO	World Health Organization
WT	Wild Type

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

The following chapter intends to inform the reader regarding the current presence of Chlamydia within our modern society as well as further educate about *C. trachomatis* including its life cycle and invasion mechanism. The hypothesis of this paper is that when Wild Type (WT) Tarp, a protein which is thought to play a significant role in facilitating the invasion of *C. trachomatis* into host cells, is expressed alongside of a mutant Tarp protein that has one of its five key domains removed, the rate of invasion for *C. trachomatis* will be reduced. This was observed with both the removal of the Actin Binding Domain (ABD) and the Phosphorylation Domain. This background information is key to understanding why the experiments in chapter 2 were performed and why these results are significant.

1.1 The Genus *Chlamydia* Includes Many Unique Parasitic Bacteria

Chlamydia are a genus of gram negative bacterium that act as obligate intracellular parasites (Figure 1). *Chlamydia* is immediately recognized as a human sexually transmitted disease; however, the *Chlamydia* genus encompasses more than just a widespread STD. There are currently known to be three separate species of chlamydia able to infect humans, *Chlamydia psittaci, Chlamydia pneumoniae,* and *Chlamydia trachomatis* [1,2]. While *Chlamydia psittaci* and *Chlamydia pneumoniae* are both known for causing respiratory infections, neither one is considered to be as financially destructive as *Chlamydia trachomatis*.

1.1.1 *Chlamydia trachomatis* Epidemiology

One of the most prevalent bacteria among the *Chlamydia* genus affecting man is the species *Chlamdyia trachomatis.* In 2008, according to the World Health Organization, 25 million adults within the Americas, 17 million adults within Europe, and 100 million adults worldwide at any given point in time were infected by *C. trachomatis* [3]. There has also been a reported 4.1% increase in the number of

new cases reported across the world, rising to 105.7 million new cases in 2008 from 101.5 new cases in 2005. In addition, it was estimated that a total of 2.86 million cases occur annually within the United States [4]. Finally, *C. trachomatis* has risen as the most frequently reported bacterial STD within the United States with 1,441,789 cases reported to the CDC in 2014 [5]. We have also observed that *C. trachomatis* is the world's leading source of curable blindness with 3% of all cases of blindness worldwide being due to an infection with the bacterium.

C. trachomatis is further divided up into different sub-species, otherwise known as serovars, based on the various surface antigens that are present in its outer membrane [6]. In total, there are fifteen different *C. trachomatous* serovars: A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, and L3. The first four of these serovars are primarily responsible for causing an eye infection known as trachoma [1]. Serovars D-K are primarily responsible for urogenital infections within humans [1]. Finally, serovars L1, L2, and L3 are responsible for causing lymphogranuloma venerium in humans [1].

1.1.1.1 *C. trachomatis* and Trachoma

C. trachomatis serovars A-C are primarily responsible for a form of eye infection known as trachoma. These infections have mostly disappeared from the first world and instead are only largely present in the third world and poor rural areas where living conditions are more unhygienic due to a lack of things such as clean water [1,7]. This form of infection primarily affects small children ages one through nine, though it is possible for adults to contract the disease [7]. It is important to note that due to the nature of trachoma infection, it is not considered to be an STD. There are two primary ways that trachoma can be spread: through direct contact with an infected source, such as another infected eye or an object covered in the bacteria, or from person to person through an insect vector, namely flies [8]. Multiple lengthy eye infections with these serovars of *C. trachomatis* results in trichiasis, a condition where the eyelashes of the afflicted turn inwards and then cause severe scarring of the cornea every time

the victim blinks. Ultimately, this scarring causes irreversible damage to the eye resulting in permanent blindness [7,9].

1.1.1.2 *C. trachomatis* and Urogenital Infections

Serovars D-K of *C. trachomatis* are primarily responsible for causing urogenital infections and are thought to be the most common serovars among those infected with the bacterium [1]. Unlike serovars A-C, these forms of Chlamydia are considered to be STDs and are spread primarily through sexual contact with an infected individual. This sexual contact can be through oral, vaginal or anal means. A *C. trachomatis* infection can lead to a variety of symptoms including nongonococcal urethritis and proctitis as well as cervicitis in females and epididymitis in males. Chronic infections can lead to even worse symptoms including inflammation, scarring, and pelvic inflammatory disease which can lead to infertility and ectopic pregnancy [1, 10, 11]. Finally, this STD form of *C. trachomatis* can be transferred from an infected mother to her newborn child through direct contact with infected tissue. This infection can result in neonatal conjuctivits or pneumonia [1].

1.1.1.3 *C. trachomatis* and Lymphogranuloma Venerium

The final three serovars of *C. trachomatis*, serovars L1, L2, and L3, are responsible for a deep tissue disease known as lymphogranuloma venerium. This infection is targeted to submucosal tissues and lymph nodes and will also target monocytes and macrophages for infection [1, 12]. Normally, symptoms of this disease include the formation of a self-limited genital ulcer at the initial site of infection as well as lymphadenopathy; however, infections can be much more dangerous and cause a systemic infection that results in chronic colorectal fistulas and strictures or reactive arthropathy [12].

1.1.2 *Chlamydia pneumoniae*

Chlamydia pneumoniae is another member of the *Chlamydia* genus that is able to infect humans. Specifically, *C. pneumoniae* is able to infect the respiratory tract of humans through inhalation of droplets that contain the bacterium. While it is expected that many within the world's population are exposed to *C. pneumoniae* on a regular basis, it is usually only the young, children between the ages of five and fourteen, that become infected with the disease [1, 13]. Those infected individuals will then be able to act as a reservoir and spread the disease to other individuals. Individuals infected by the disease are normally asymptomatic but can show symptoms including bronchitis and pneumonia [1, 14].

1.1.3 *Chlamydia psittaci*

Chlamydia psittaci is a zoonotic disease that mainly infects birds including parrots, parakeets, and canaries. While birds are the primary targets of this form of *Chlamydia*, it is also possible for this bacterium to be transferred from infected birds to humans. The bacterium is released into the air through small droplets from their urine, feces, or respiratory secretions or through direct contact with the infected animal [15]. Once inhaled, the bacterium will come to inhabit the host's lung epithelial cells and will eventually cause psittacosis in the infected host [15]. Psittacosis is any infection caused by the bacterium *C. psittaci*. This form of infection most typically manifests itself as inflammation of the lungs and an atypical pneumonia but can also spread throughout the body and affect multiple other organs including the heart, liver, and intestines. A psittacosis infection carries with it a small chance of multi-organ failure and, ultimately, death but most infected will just appear to have mild flu like symptoms until the infection clears or is treated [15].

1.1.4 Treatment of *Chlamydia*

C. trachomatis and other members of the *Chlamydia* genus are normally treated with antibiotics such as azithromycin and doxycycline once it has been identified as present within an infected patient [1, 7, 14]. Most of the time, a patient must be diagnosed as actually being infected before treatment is offered to them. This presents a unique problem in that many cases of *Chlamydia* infection are asymptomatic. In fact, the CDC has estimated that as few as 10% of men and 30% of women actually develop symptoms despite having a clinically confirmed infection of *C. trachomatis*. In the future, through the further development of Polymerase Chain Reaction (PCR) technology and use of bedside thermocyclers in diagnosing common ailments in clinics and hospitals, it may become more feasible to screen every individual for a *Chlamydia* infection and treat them accordingly. In addition to this, the World Health Organization(WHO), has created a different system to treat the non-STD form of *C. trachomatis* that is able to infect individual's eyes and cause trachoma. This system is known as the SAFE strategy and includes the components of Surgery for trichiasis, Antibiotics, Facial cleanliness, and Environmental improvement. Using these angles of treatment and prevention, the WHO hopes to eliminate the threat of trachoma throughout the world by the year 2020 [1, 7, 9].

1.2 The Developmental Cycle of *C. trachomatis*

C. trachomatis, as well as all other Chlamydia species, are obligate intracellular parasites that must invade a eukaryotic host cell to be able to both survive and proliferate [16]. *C. trachomatis* specifically targets human epithelial cells and, as previously described, will invade a particular tissue within the body based on its serovar [1, 2]. When not inside of a host cell, *C. trachomatis* exists in a spore-like, partially metabolically active state commonly identified as an elementary body (EB) [17]. While in this form, the EB is neither able to divide nor is it able to produce the additional components necessary for chlamydial replication. Instead, the EB acts as a pre-packaged infectious unit that is able to

secrete effectors into a host cell to cause the cell to engulf the EB into a membrane bound vacuole known as an inclusion. Once inside of a host cell, the EB will be converted into a fully metabolically active form known as a reticulate body or RB. This form will be able to undergo binary fission to produce more RBs. The RBs will then differentiate back into EBs prior to release back into the environment where they will be able to seek out a new host cell and begin the process anew. This life cycle is known as a biphasic life cycle due to the two major components that it contains [17] (Figure 2).

1.2.1 The Elementary Body

C. trachomatis is traditionally depicted as beginning its developmental cycle in the form of a small infectious unit known as the elementary body. The EB is usually 0.3 μM in size and is most easily thought of as a spore-like structure that serves two primary purposes. The first purpose is to protect the EB from the environment around it. EBs are highly resilient to damage as indicated by their ability to resist being lysed when placed under duress such as being sonicated or osmotic stress. The second major feature of the EB is that it renders the bacterium partially metabolically inert. This form allows the EB to survive for a longer period of time while outside of a host cell by allowing it to only consume a minimum amount of its pre-packaged resources. Both of these features are achieved through the creation of disulfide cross-linked protein complexes by the bacterium [18]. It was long thought that the bacterium, when in its EB state, was entirely metabolically inert but new evidence has shown that this may not be the case for all species within the genus. *Protochlamdyia ameobophila*, a species of chlamdyia that primarily infects amoeba, has been proven to be able to uptake extracellular phenylalanine as well as an ability to express 472 proteins when in its EB state [19, 20]. EBs of the *Chlamydia* genus have also been shown to contain a set of proteins that allows them to transcribe proteins and synthesize DNA [21]. While it was originally thought that these proteins were pre-packaged into the EB to allow for the immediate invasion of a new host cell, it stands to reason in the face of evidence from *P. ameobophila* that these proteins may

also allow *Chlamydia* EBs to have some level of metabolic activity. Regardless of its metabolic activity, the EB is able to serve as an effective infectious unit in harsh extracellular environments and, once the EB form of *C. trachomatis* comes into contact with a suitable host cell, the process of invasion will begin [22].

1.2.2 Invasion into a Host Cell

After finding an appropriate host, the *C. trachomatis* EB will begin the process of invading the host cell. The ability to recognize and adhere to a host cell is one of the most important abilities of an EB due to the fact that it is an obligate intracellular parasite. The adhesion and uptake of an EB into a host cells is thought to be a two-step process. This process begins when OmcB, a protein within the Chlamydial Outer Membrane Complex (COMC) makes contact with a host cell, allowing the bacterium to determine that a suitable host has been found and then to adhere to that cell using the glycosaminoglycan (GAG) heparan sulfate (HS) [22, 23]. It is currently unknown if this GAG is attached to the EB before invasion occurs or if HS that has been produced within the host golgi apparatus is hijacked to facilitate the uptake of the EB. This process is considered to be a reversible electrostatic interaction. After this initial interaction, adhesins on the surface of the EB will irreversibly bind to surface receptors on the host cell. These adhesins include prominent proteins within the COMC such as the Major Outer Membrane Protein (MOMP) and a wide array of Polymorphic Outer Membrane Proteins (PMPs) [24]. From here, it is then thought that the Type III Secretion System, a needle-like delivery system on the surface of many gram negative bacteria, is able to make contact with the cell's surface and will become active [25]. This activation will allow the secretion system to begin delivering protein effectors that have been stored inside of the EB directly into the cytosol of the host cell.

1.2.2.1 Secreted Effector Proteins

There are known to be multiple separate secreted effector proteins that are stored within a *C. trachomatis* EB. Each one is known to come through the type III secretion system and are thought to play some role in the invasion of *C. trachomatis* into a host cell or development of *C. trachomatis* within an inclusion. At least four of these effectors are believed to have a defined role within *C. trachomatis*. The first, and most well-known, of the effector proteins is the Translocated Actin Recruiting Phosphoprotein (Tarp). This protein is thought to play some role in the reorganization of the host's actin cytoskeleton [26]. The second effector protein is CT166 which is thought to inhibit the activity of the Rho-protein Rac1 through glucosylation which induces host actin cytoskeleton rearrangement through the loss of key structures such as stress fibers, lamellipodia and filopodia [27]. The third effector protein is CT694 which is thought to associate with the host protein AHNAK. AHNAK is a protein that plays a role in host cell actin cytoskeleton rearrangement and affects the formation of stress fibers by the host cell, and also associates with the membrane of the host cell [28, 29, 30]. The fourth effector protein is CT875 otherwise known as the Translocated early phosphoprotein (TepP). TepP is thought to act as a regulator of multiple essential signaling pathways. This protein is tyrosine-phosphorylated upon association with host cells and then is believed to be able to recruit the host cell scaffolding proteins CrkI-II. Through the recruitment of these proteins, it is thought that TepP is able to manipulate Crk-dependent signaling functions within the host cell and regulate its innate immune response to a Chlamydia infection [31].

1.2.2.2 The Translocated Actin Recruiting Phosphoprotein

The Translocated Actin Recruiting Phosphoprotein, otherwise known as Tarp, is one of three effector proteins secreted from *C. trachomatis* that are thought to play a strong role in the bacterium's ability to invade host cells. Tarp is pre-packaged into an EB, and upon attachment to a host cell, Tarp is known to be brought to the type III secretion system by the chaperone SLC-1 where it can then be

inserted directly into the cytosol of the host cell by the needle-like delivery system [18, 32]. Tarp is specifically associated with its ability to recruit actin within host cells and is considered to be a bacterial nucleator of actin within the host cell [26].

The Tarp molecule within serovar L2 *C. trachomatis* is a molecule that is roughly 105 kiloDaltons (kDa) in size and contains 1005 amino acids. There are three distinct domains within Tarp that have unique functions: an N-terminus domain that is phosphorylated by tyrosine kinases hijacked from the host cell, a proline rich domain (PRD) that allows the Tarp molecule to oligomerize and nucleate actin, and an C-terminus domain made up of three wasp homology 2 binding domains, one defined as the actin binding domain which allows for binding to both G- and F-actin and two defined as F-actin binding domains (FAB) which allows for binding to F-actin only (Figure 3) [26, 33, 34, 35].

1.2.2.3 The Arp2/3 Complex

While Tarp has shown an ability to nucleate actin, there are additional host factors that are required to allow for EBs to successfully invade a host cell. One such host factor that is required to be activated is the Arp2/3 complex [30]. This is normally achieved through the activation of Rho-family GTPases by Tarp. These GTPases will then go on to activate the complex. Arp2/3 is another actin nucleator that focuses around creating new actin filaments that branch off of existing linear filaments [16, 36]. It is thought that the nucleation ability of Arp2/3 works in concert with the actin nucleating ability of Tarp, which is able to form actin filaments but will not form them branching off of existing filaments. These two functions together are speculated to be necessary for EBs to be taken up into a host cell [16, 36].

1.2.3 The Reticulate Body

Upon entry into a host cell, the EB will begin differentiating into a new form known as a reticulate body (RB). The RB is considered to be the fully metabolically active form of chlamydia and is where a vast majority of gene transcription and translation is performed. An RB is able to undergo the process of binary fission and is the form in which replication of *C. trachomatis* occurs while inside of an inclusion. The RB itself will be anchored to the membrane of the inclusion where it is able to secrete additional proteins into the host cell's cytosol to allow for further manipulation of the host cell [37]. The process of differentiation into a RB begins immediately upon entry into a host cell and will last for roughly 24 hours before the RB will detach from the inclusion membrane and begin to be converted back into an EB [37].

1.2.4 Development within a Host Cell

The beginning of the conversion from an EB into an RB starts immediately after the EB first enters a host cell and begins with the disulfide cross links in the COMC breaking down and the condensed DNA inside of the EB being converted into free chromatin to allow for the transcription of key genes on the bacterium's genome [37, 38]. Expression of genes within a RB can be split into three separate categories across the 24 hour period that the RB is present within the inclusion. These three stages are defined as early cycle, mid-cycle, and late cycle. The early cycle begins at or before the first two hours after invasion of a host cell. This set of genes is expressed to allow for production of proteins that will allow for the biosynthesis and processing of new proteins. For example, the α subunit of DNA polymerase is produced by the transcription of the gene *DnaE* in this phase [37, 38]. The mid-cycle begins between six and twelve hours after invasion of a host cell. The proteins expressed within this stage are thought to be expressed to allow the RB to operate its metabolism as well as keep both it and the inclusion that it inhabits stable while the bacterium grows and undergoes binary fission. The genes

expressed within this stage include *ompA* which encodes the major outer membrane protein and *incA* which expresses a protein that allows multiple inclusions that may have formed within the host cell to merge into a single inclusion [37, 38]. The late cycle begins 18 hours after invasion of a host cell. The proteins expressed within this final stage are thought to be produced to convert an RB back into an EB as well as any proteins that will need to be pre-packaged into the newly formed EB so that it can invade a new host cell once it is released into the extracellular environment. Genes expressed at this stage include *htcA*, a histone-like protein, and *tarP*, which produces the Tarp protein [37, 38].

1.2.5 Egress from a Host Cell

After the 48 hour life cycle of *C. trachomatis* has transpired, the newly formed EBs that are inside of the inclusion will have to be released into the surrounding environment so that they can seek out a new host cell and begin the process anew [37]. This process of egress can occur in one of two ways. The first is that cysteine protease induced cell lysis can occur causing the host cell, which is now primarily taken up by the inclusion, to burst open by having its membrane ruptured [39]. The other mechanism is that the inclusion can be pushed out of the cell and its contents can be released into the surrounding environment. This is done through N-wasp polymerizing actin and then rearranging the cytoskeleton so that the inclusion can be pushed to the edge of the cell and it can then fuse with the membrane [40]. In either case, only the newly formed EBs will be able to survive in the extracellular environment and find a new host cell. Those RBs that were not able to convert will not be able to survive without a host cell. It is not known if the second form of egress is possible in a true *in vivo* infection of human epithelial cells with *C. trachomatis* as it has only been observed *in vitro*.

1.3 The Chlamydial Outer Membrane Complex

Due to its status as an obligate intracellular parasite, *C. trachomatis* requires a wide array of proteins to be able to mediate its various functions such as recognizing potential hosts or switching between metabolically active and metabolically inert. Many of the proteins that mediate these functions are located within the Chlamydia Outer Membrane Complex [18]. The COMC is a lattice of proteins made up primarily of a protein known as the Major Outer Membrane Protein as well as the two proteins OmcA and OmcB. One other group of important proteins within this complex is the Polymorphic Outer Membrane Proteins. All of these proteins are able to crosslink together using disulfide bonds to allow for the formation of an EB. These disulfide bonds can then be broken so that the COMC can be taken apart once an EB enters a host cell and begins to be converted into an RB [18].

1.3.1 OmcA and OmcB

OmcA and OmcB are two prominent lipoproteins within the COMC of *C. trachomatis* [18]. While they are both located in the same structure, each one of these proteins plays its own distinct role in *C. trachomatis*. OmcA is a cysteine rich 12 kDa protein that is speculated to be one of the proteins that allow an EB to retain its shape [18]. It is also speculated that OmcA's expression is key to allowing an RB to be converted back into an EB. OmcB is a 60 kDa protein that is also cysteine rich. OmcB has a domain that is able to bind to heparin and thus it is speculated to be an adhesin that may allow for the uptake of *C. trachomatis* into a host cell [18, 22, 41].

1.3.2 Major Outer Membrane Protein

One of the most important proteins in the COMC is the Major Outer Membrane Protein. Thought to be an adhesin, the MOMP is considered to be the most important protein within the cell envelope of *C. trachomatis* due to the sheer percentage of proteins that are MOMP within both stages of the bacterium

[42]. Within its EB state, the COMC is made up of 60% MOMP and within its RB state, the COMC is made up of between 90 and 100% of MOMP [18, 22, 42, 43]. It is also thought that MOMP plays a role in the ability for an EB to invade a host cell as when these proteins were blocked using serovar specific antibodies, the EBs showed no ability to invade host cells [44]. MOMPs cross link with one another along with the membrane proteins OmcA and OmcB using disulfide bonds to allow for the formation of the EB and, when the cell is converted into an RB, those disulfide bonds are broken allowing for the new form to take shape [37].

1.3.3 Polymorphic Outer Membrane Proteins

Polymorphic Outer Membrane Proteins are a set of type V autotransporters that are secreted across the inner membrane of *C. trachomatis* [18, 45]. There are currently known to be nine separate PMPs expressed within *C. trachomatis*. The function of all but one of these PMPs is currently unknown. PmpD is the only protein to have a known function and has been shown to be an adhesin [45].

1.4 Development of Genetic Tools for *C. trachomatis*

The genes within *C. trachomatis* have traditionally been studied *in vitro* due to the fact that no transformation system for *C. trachomatis* had ever been successfully developed; However, it is currently known that a plasmid resides within most *C .trachomatis* serovars which would suggest that a plasmid based expression system should allow for expression of altered proteins to be studied *in vivo* and would allow for pieces of the bacterium's genome to eventually be replaced [46]. The development of this system should prove key in furthering our understanding of the role that the Tarp protein plays in *C. trachomatis*'s life cycle. It could also be used to further elucidate the roles that all other proteins expressed on the bacterium's genome play in its invasion, development, and egress from host cells.

1.4.1 Plasmids Naturally Within *C. trachomatis*

There is currently known to be one plasmid that natively resides within *C. trachomatis* [46]. This 7.4 kb plasmid is thought to act as an additional virulence factor within the lifecycle of *C. trachomatis*. Through previous *in vivo* studies, the *C. trachomatis* samples which contained this plasmid were more likely to be uptaken into the epithelial cells of a mouse female genital tract than *C. trachomatis* which did not contain the plasmid [47]. It is unknown if the plasmid plays any true role *in vivo* as the only different phenotype that is observed between those samples that have the plasmid and those that do not are that those bacteria that do contain the plasmid are able to form glycogen granules in their host cells. In addition to this speculation, the plasmid also demonstrates to us that *C .trachomatis* can successfully harbor a plasmid and use it to express proteins.

1.4.2 Initial System Developed

A transformation system for *C. trachomatis* has been long thought about within the scientific community, and many attempts have been made to transform plasmid DNA into a *C. trachomatis* EB including trying to use electroporation to introduce a chimeric plasmid into the bacterium in 1994 [48]. However, an effective transformation system was not fully realized until developed in Dr. Ian Clarke's lab in 2011 with the successful use of a transformation system using calcium chloride [49]. After discovering that *C. trachomatis* is naturally able to maintain a plasmid, it was thought that a new plasmid could be placed directly into purified EBs. This plasmid could be used to directly replace key regions of the genome to allow for the further elucidation of function of proteins thought to play a role in the invasion, development, and egress of the EB. In addition, the EBs that took up the plasmids could be selected for through the use of a penicillin resistance gene, and it could be proven that they had taken up the plasmid when they expressed Green Fluorescent Protein (GFP). These GFP expressing plasmids were

placed into a *C. trachomatis* strain that did not naturally have a plasmid to ensure that the inserted plasmid would be the only outside factor affecting the genome.

1.4.3 Development of Transformants

Using this same transformation system, the Jewett lab was able to successfully create five separate plasmids expressing a mutant form of Tarp and then place these plasmids into the L2 serovar of *C. trachomatis*. Four of these plasmids are missing a key Tarp domain including the phosphorylation domain, the proline rich domain, the actin binding domain, and the two F-actin binding domains. A fifth plasmid was also created to serve as a control where the entire Tarp protein is being expressed. Each of these Tarp proteins also contains a C-myc tag which will allow us to identify the presence of these transformant proteins in future experiments.

1.5 Hypothesis

Through the use of the plasmids our lab has developed, we hypothesize that these plasmids will be able to be successfully transformed into L2 *C. trachomatis* and that they will allow for the expression and subsequent transport of Tarp missing key domains into the host cell cytosol alongside Wild Type (WT) tarp expressed from the genome of these transformants. In addition, we hypothesize that the Tarp protein plays a key role in *C. trachomatis's* ability to invade a host cell and that through its alteration and subsequent expression within a transformant *in vivo*, the invasion phenotype of these transformants will be altered. Primarily, we expect those transformants that express Tarp without an Actin Binding Domain will have a decrease in their invasion phenotype based on previously observed *in vitro* pyrene assays performed. In addition to this, we expect that there will be no effect on the growth phenotype of these transformants as Tarp has not been shown to play a role in *C. trachomatis'* ability to develop within an inclusion.

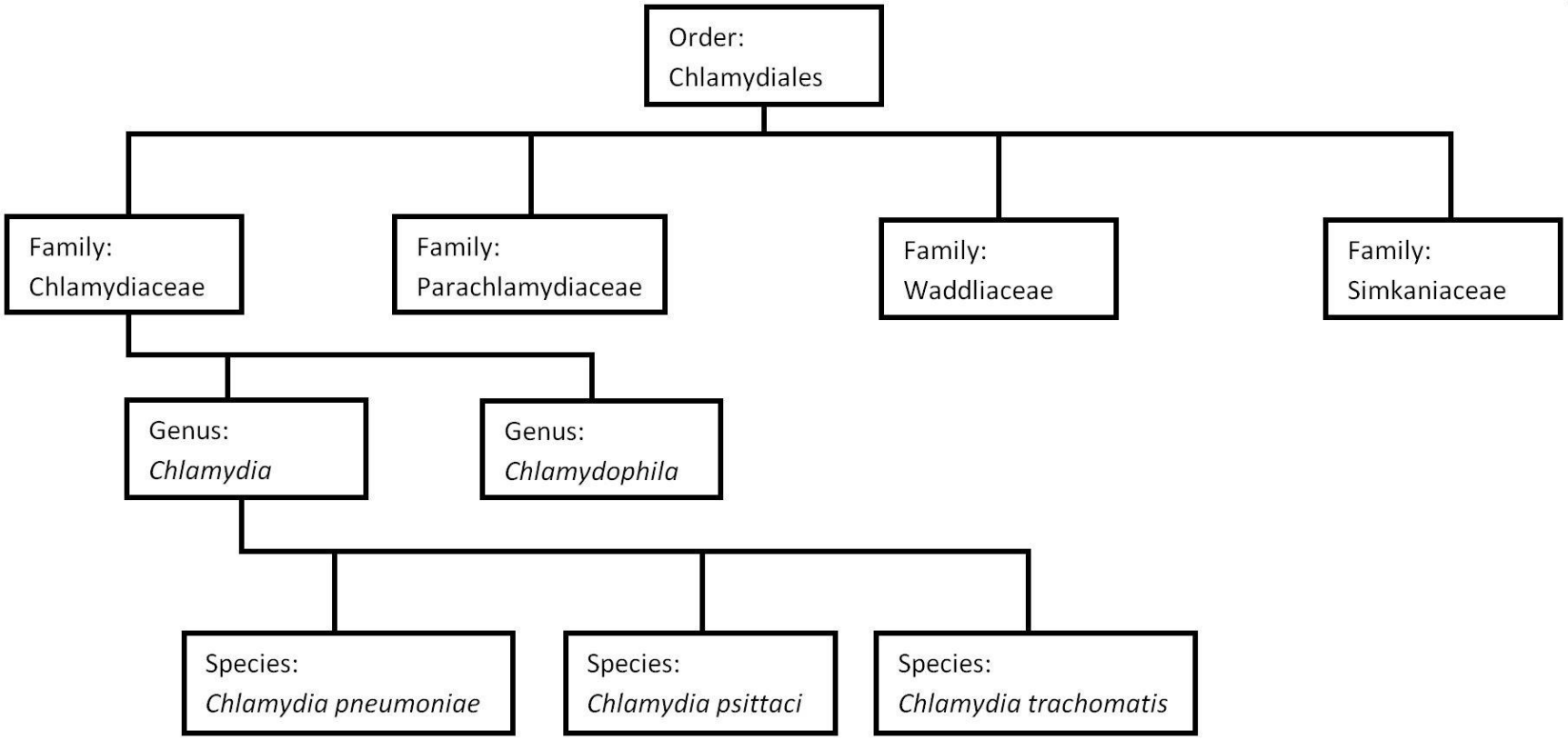

Figure 1: Taxonomic Tree of the Order Chlamydiales

The order, Chlamydiales, is comprised of four separate main families: Chlamydiaceae, Parachlamydiacae, Waddliaceae, and Simkaniaceae. *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia trachomatis* are all species within the *Chlamydia* genus which is a member of the Chlamydiaceae family.

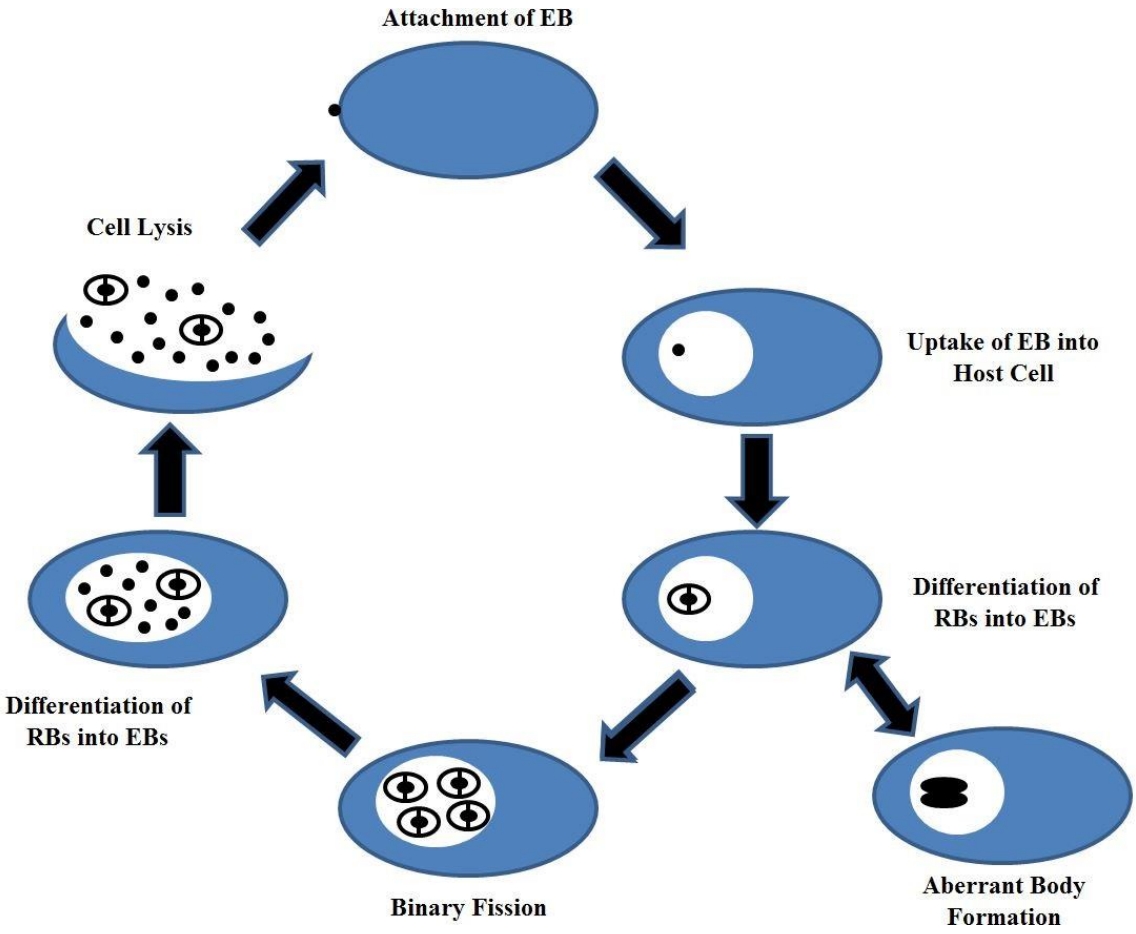

Figure 2: The Biphasic Developmental Cycle of *Chlamydia trachomatis*

Chlamydia trachomatis has a biphasic lifecycle. It begins outside of a host cell as a partially metabolically active EB and then attaches itself to a host cell. Once attached it is taken up into the host cell in a formation known as an inclusion where it is then converted into a fully metabolically active form known as an RB. The RB then undergoes binary fission to multiply within the inclusion. As more and more RBs are made the inclusion begins to grow and overtake the host cell. The RBs will begin to convert back into EBs when the cycle is starting to finish. Finally, the number of RBs and EBs within the inclusion becomes too much to be contained within the EB and it bursts open, releasing both forms into the extracellular environment. The RBs will die while the EBs seek out a new host cell and the cycle begins anew.

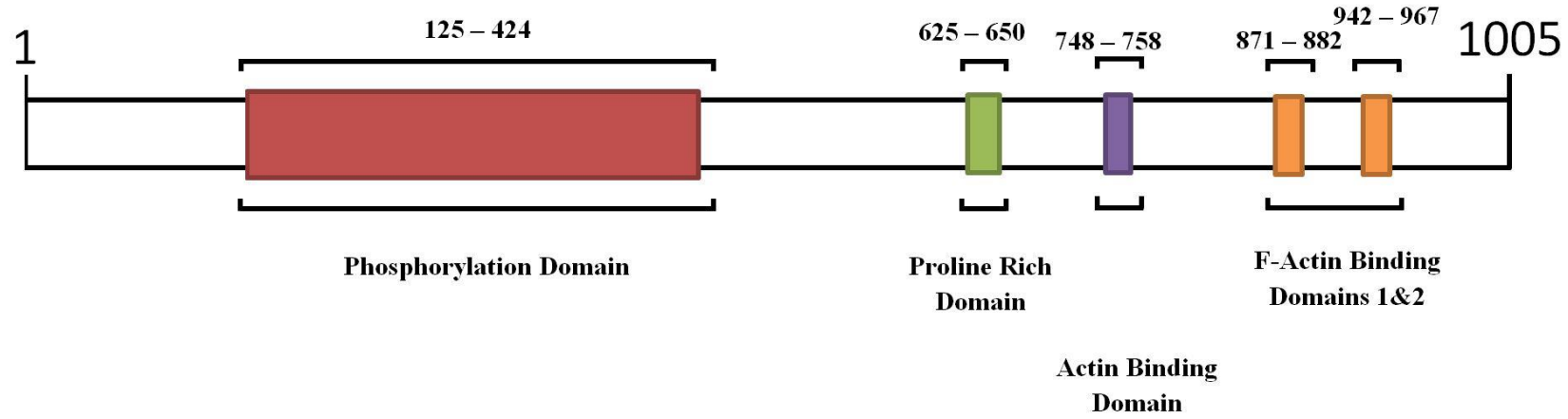

Figure 3: The Tarp Molecule of *Chlamydia trachomatis*

The Tarp molecule of *C. trahcomatis* is currently known to be comprised of five separate domains including the Phosphorylation domain, the Proline Rich domain, the Actin Binding domain, and two F-Actin Binding domains.

CHAPTER 2: TARGETED DISRUPTION OF *CHLAMYDIA TRACHOMATIS* INVASION BY DOMINANT NEGATIVE TARP EFFECTORS

2.1 Introduction

The genus *Chlamydia* is made up of a diverse set of obligate intracellular parasites that are able to cause a wide variety of human diseases [1]. Of particular note within this genus is the species *Chlamydia trachomatis*. This species is made up of multiple serovars including A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, and L3. Using these serovars, *C. trachomatis* is able to cause the formation of eye infections known as trachoma, urogenital infections, and lymphogranuloma venerium [1, 2, 12]. *C. trachomatis* have a unique biphasic lifecycle that allow them to survive in harsh environments while outside of a host cell and then switch into a metabolically active state when inside of a host cell so that they can undergo binary fission to further expand within their environment. The bacterium will start off in the metabolically dormant Elementary Body (EB) form until it comes into contact with a host cell. The EB will then enter the host cell where it will differentiate into its metabolically active form known as the Reticulate Body (RB). In this form, roughly 24 hours after entering a host cell, the RBs will be able to undergo a process known as binary fission to expand its numbers. The RB will then begin to differentiate back into its EB form so it can survive when released back into the environment and find a new host cell to begin the process anew [37].

One of the most important features that allow for the invasion of host cells is the Type III secretion system. This system is found within gram negative bacteria and allows for the transfer of effectors from inside of the bacterium directly into the cytosol of the host cell [30, 32]. Once *C. trachomatis* makes contact with a host cell, it is known that there are at least three early effectors secreted from the EB [30]. One of the most important effectors is the Translocated Actin Recruiting

Phosphoprotein, otherwise known as Tarp. The Tarp molecule within *C. trachomatis* is made up of five distinct regions, a C-terminal Phosphorylation Domain, a Proline Rich Domain (PRD), an Actin Binding Domain (ABD), and two N-terminus F-Actin Binding Domains (FAB) [33, 34, 35]. The *tarP* gene is known to be in all current clinical isolates of *Chlamydia*, however, *C. trachomatis* Tarp is slightly different from any other known form of Tarp due to the fact that it has the C-terminus Phosphorylation Domain [33]. This first domain is tyrosine phosphorylated by host kinases upon entry into the host cell. The second domain within Tarp is the Proline Rich Domain which is responsible for allowing the Tarp proteins, once inside of the host cell, to be able to oligomerize into a multimer. The third domain within Tarp is the Actin Binding Domain which is responsible for allowing Tarp proteins to bind to both filamentous actin and actin monomers. The fourth and fifth domains within Tarp are two F-Actin Binding Domains and they allow Tarp to also bind to filamentous actin [35]. Through the ability to bind to both F- and G-actin granted by both the Actin Binding Domain and the two F-Actin Binding Domains, the Tarp molecule has the ability to bind to and bundle actin filaments as well as nucleate globular actin. These actions allow the actin cytoskeleton of the host cell being invaded to be re-arranged turning it into a phagocytic cell. The cell is then forced to form a vesicle around the EB attached to it, internalizing the bacteria in a formation known as an inclusion.

Despite all of our knowledge about the Tarp molecule, none of the data has ever been able to be confirmed *in vivo* and instead has been gained entirely through previously performed *in vitro* experiments. The Jewett lab sought to further define the role of the Tarp molecule *in vivo* by generating mutant Tarp molecules that would be expressed within *C. trachomatis* on a plasmid expression system. Each plasmid generated was able to express either a Tarp molecule missing one of its five key domains with a c-myc tag or a Wild Type (WT) Tarp molecule that was expressed with a c-myc tag. In our results we observed that two major transformants were able to cause a statistically significant decrease in the invasion phenotype of *C. trachomatis*. The transformant which was missing the Actin Binding Domain

was predicted to have a drop in its rate of entry by our hypothesis and our results confirmed this. In addition, the transformant which was missing the Phosphorylation Domain showed the largest decrease in the invasion phenotype. This is surprising because previous *in vitro* data seemed to suggest that the phosphorylation domain had no role in the process of invasion for *C. trachomatis*. Ultimately, this data suggests that Tarp is an essential virulence factor in *C. trachomatis* and plays a significant role in its pathogenicity.

2.2 Materials and Methods

2.2.1 *Chlamydia trachomatis* Serovar and Purification

All *Chlamydia trachomatis* used were of the L2 serovar (LGV 434) and purified through the use of Renografin density gradient centrifugation [50] after being grown in McCoy B cells (ATCC CRL-1696) for 48 hours.

2.2.2 Cloning and Transformation of *Chlamydia trachomatis*

In previous studies we had generated a number of in-frame Tarp deletions which were expressed as mutant GST-Tarp fusion proteins from pGEX-6p-1 (GE Health Sciences) plasmids [35]. Tarp domain deletion mutants included: phosphorylation domain deletion (Δphos; deletion of D125 to Y424), proline rich domain deletion (ΔPRD; deletion of S625 to N650), actin binding domain deletion (ΔABD; A748 to K758), and F-actin binding domain 1 & 2 deletion (ΔFAB 1&2; deletion of L871 to G1005). These mutant Tarp alleles were subcloned into the chlamydial shuttle vector pCTSV.1 in a two-step process. First, wild type Tarp sequence was amplified from *C. trachomatis* (LGV 434) genomic DNA (Qiagen genomic purification kit, Valencia, CA). The forward (5'ACTCCGCGGTATTGCATTTCTTCACAAACGTTACC-3') and reverse (5'TATATACAATTGTTACAGGTCCTCTTCAGATATTAGTTTTTGTTCTCCTACGGTATC

AATCAGTGAGC-3') DNA primers (Integrated DNA Technologies, Coralville, IA) were engineered to amplify 200 bases of putative Tarp promoter sequence and an in frame c-myc epitope tag by PCR with SacII and MfeI linkers. PCR products were purified (Qiagen), digested with restriction enzymes (New England Biolabs, Beverly, MA) and cloned into linearized pCTSV.1. This procedure resulted in the parent pCtSV.Tarp plasmid in which all other plasmids engineered to express Tarp mutants may be generated. pCtSV.Tarp mutant derivatives were generated by exchanging the mutant DNA sequence from those pGEX-6p-1 clones described above. For example, pCtSV.Tarp Δphos resulted from DNA exchange with digested Tarp DNA sequence flanking the phosphorylation domain with restriction sites BstAP1 and BmgB1 from pGEX-6p-1 Tarp Δphos. Similarly, the other pCtSV.Tarp mutant clones were generated albeit with unique restriction enzymes which flanked the corresponding domain: The proline rich domain with BmgB1 and Bsm1, the actin binding domain with Bsm1 and Nco1, and the F-actin binding domains 1&2 with Nco1 and Mfe1. All engineered vectors were confirmed to be free of extraneous mutations by DNA sequence analysis and all in frame domain deletions were verified. All chlamydial shuttle vectors were purified from *E. coli* K12 ER2925 cells (New England Biolabs) and transformed into *C. trachomatis* (LGV 434) as described by Wang et al.,[49]. Briefly, 20 μg of plasmid DNA was mixed with 1 x 108 density gradient purified *C. trachomatis* EBs in 500 μL of 50mM CaCl2 10mM Tris pH 7.4 for 30 minutes at room temperature. Following the room temperature incubation, EBs and DNA were added to three T175s containing McCoy cells at 60% confluency. Chlamydial development proceeded in the presence of 7 μg/ml of penicillin and drug resistant EBs were purified from infected cells every 48 hours (one developmental cycle) and blindly passaged onto fresh host cells to increase the inclusion forming units (IFUs).

2.2.3 Pyrene Assays

Pyrene actin polymerization assays were performed as previously described [35]. Briefly, monomeric pyrene-labeled actin was prepared by diluting 100 μg of lyophilized pyrene actin (cytoskeleton Inc. Denver, CO) in 2mL of 5mM Tris (pH 8.0), 0.2mM CaCl2, 0.2mM ATP (G buffer) and incubated for 1 hour at room temperature, followed by an additional 1 hour incubation at 4^{o}C. Monomeric pyrene actin was obtained by collecting the supernatant after a 2-h 100,000 x g 4°C spin in a Beckman Optima TLX Ultracentrifuge using a TLA 100.3 rotor (Beckman Coulter). Approximately 20 μg of pyrene-labeled actin was gently mixed with 5 μg of GST fusion proteins in a volume of 500 ml for 10 min before the addition of 1/20th volume of polymerization buffer (500 mM KCl, 20 mM MgCl2, 10 mM ATP). The reaction was monitored over 1 hr with an LS 55 Luminescence spectrophotometer directed by FL WinLab software version 4.0 (Perkin-Elmer, Beaconsfield, Bucks, United Kingdom) with 2.5-nm bandwidth at 365-nm excitation wavelength and 2.5-nm bandwidth at 407-nm emission wavelength.

2.2.4 SDS-PAGE and Immunoblotting

Proteins were separated on 5 to 15% SDS polyacrylamide gels (BIORAD, Hercules, CA) and transferred to 0.45-μm pure nitrocellulose transfer and immobilization membranes(Schleicher & Schuell, Keene, NH) or stained with Imperial protein stain (Pierce, Rockford, IL) . Primary antibodies used include anti-actin C4 monoclonal antibody (Chemicon International), anti-actin polyclonal antibody (Cytoskeleton, Inc.), anti-phosphotyrosine 4G10 monoclonal antibody from Upstate (Millipore), anti-chlamydial EB polyclonal antibody (Pierce), alkaline phosphatase conjugated primary c-myc antibodies (clone 9E10) (Sigma, St. Louis, MO), Momp monoclonal antibody (Pierce), GAPDH monoclonal antibody (Pierce), anti-c-myc monoclonal antibody (Genescript, Piscataway, NJ), and the polyclonal rabbit antibodies directed toward C. trachomatis L2 LGV 434 Tarp (CT456) were developed at Rocky

Mountain Laboratories as previously described [51]. Secondary antibodies used in immunoblotting were HRP conjugated (Chemicon International, Temecula, CA). HRP conjugated secondary antibodies were activated with Supersignal West Pico chemiluminescent substrate (Thermo Fischer Scientific, Waltham, MA) while alkaline phosphatase conjugated primary antibodies were activated using Western Blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI.)

2.2.5 Generation of Red *Chlamydia trachomatis* Transformants

Red *C. trachomatis* transformants were formed by adding CellTracker™ Red CMTPX Dye to host cells twelve hours after initial infection with a *C. trachomatis* transformant. Celltracker dye was received as a powder and was resuspended in 30 ul dimethyl sulfoxide before being added to a T-175 flask infected with a transformant and filled with 50 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% L-glutamine. The media containing the dye was removed after 12H and replaced with 50 mL fresh DMEM containing 10% FBS and 1% L-glutamine. The transformant was allowed to expand in the flask for an additional 24H before being harvested through the use of Renografin density gradient centrifugation [50].

2.2.6 Invasion Assay and Microscopy

HeLa 229 cells were seeded in 24 well plates with cover slips and grown in 1 mL DMEM containing 10% FBS and 1% L-glutamine for 24 hours prior to infection. On the day that the experiment was performed, each well was prepared for a synchronized infection by putting the plate on ice for 30 minutes. Media was then removed from each well and 200 ul of Hank's Balanced Salt Solution was put into each well. Red CMPTX-labeled *C. trachomatis* EBs were then added to each well and permitted to attach to HeLa 229 host cells for 30 minutes at 4^{o}C. Media pre-warmed to 37 ° C was then added to each well and the plate was placed into a 37 ° C 5% CO2 incubator for one hour. Cells were then fixed using a

4% paraformaldehyde solution to ensure that they were not permeabilized. Immunostaining was performed by first blocking the coverslips in one mL of a 10% fetal bovine serum solution in phosphate buffered saline (PBS) for one hour. Cover slips were then incubated in a monoclonal anti-Major Outer Membrane Protein (MOMP) antibody at a 1:50 concentration for one hour and washed five times with a cold PBS solution. Cover slips were then incubated in an alexafluor 488 conjugated secondary antibody at a 1:1000 concentration for one hour and again washed five times with a cold PBS solution. Cover slips were then mounted onto slides using ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). Cover slips were observed under a Zeiss Axio Observer A1 microscope equipped with a phase-contrast and epifluorescence optics. Images were obtained using an AxioCam MRm camera controlled by Axio Vision 4.8.2 and then processed using Adobe Photoshop CS2. The total number of red and green EBs was tallied for each cell counted and these numbers were then used to determine how many elementary bodies were internalized by the host cell. The number inside was divided by the number outside, multiplied by 100 to determine a percentage of bacterium that were inside of the cell. Twenty fields of view were taken from each cover slip and these percentages were then averaged together to determine the final invasion rate.

2.2.7 Immunoprecipitation

Chlamydia trachomatis infected McCoy cells or McCoy cells alone were removed from flasks and suspended in 100mM KCl, 10 mM HEPES (pH 7.7), 2mM MgCl2, and 2mM ATP (Buffer A) and disrupted by sonication delivered in three consecutive 30 second intervals at 30% power using an ultrasonic sonicator processor XL equipped with a microtip (Misonix Incorporated, Farmingdale, NY). Insoluble material including intact EBs was removed by centrifugation (10,000 x g; 25 min; 4^{o}C). 100 µL of anti-c-Myc agarose beads (Pierce) were incubated with each lysate. Following a 4 hour incubation at 4^{o}C, antibody coated beads and bound antigens were washed four times with buffer A and suspended in

200 μL of protein sample buffer. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with antibodies specific for c-myc, Tarp, and actin.

2.2.8 Subcellular Fractionation and Protein Extraction

Chlamydia trachomatis infected cells underwent subcellular fractionation as previously described [52]. Briefly, *Chlamydia trachomatis* infected McCoy cells maintained at 37^{o}C or 4^{o}C or McCoy cells alone incubated at 37^{o}C were removed from flasks and suspended in 100mM KCl, 10 mM HEPES (pH 7.7), 2mM MgCl2, and 2mM ATP (Buffer A) and disrupted by sonication delivered in three consecutive 30 second intervals at 30% power using an ultrasonic sonicator processor XL equipped with a microtip (Misonix Incorporated, Farmingdale, NY). All cell lysates underwent subcellular fractionation by sequential centrifugation in which supernatants and pellets were separated. Lysates were initially subject to an 800 x g spin for 15 minutes at 4^{o}C. The 800 x g supernatants were then subjected to a 10,000 x g spin for 30 minutes at 4^{o}C. The remaining 10,000 x g supernatant underwent a 100,000 x g spin for 1 hour at 4^{o}C. Protein sample buffer was added to all pellets and supernatants and proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with antibodies specific for c-myc, Tarp, actin, GAPDH, Momp, and EBs.

2.2.9 Growth Curve

HeLa 229 cells were seeded in 6 well plates and grown in DMEM containing 10% FBS and 1% L-glutamine for 24 hours prior to use. Five individual wells were then infected with WT *C. trachomatis* (LGV 434) or a *C. trachomatis* transformant. Wells were harvested (cells scraped off bottom of well using P1000 tip, collected in 15 mL conical tube, and sonicated at 20% power for 30 seconds using a 1/64 mm tip attached to a Misonix sonicator) at 0, 12, 24, 36, and 48H. Harvested material was then frozen at -80 ° C until all time points had been collected. Material was thawed on ice and then placed on HeLa 229

cells grown in 24 well plates with cover slips and DMEM containing 10% FBS and 1% L-glutamine in triplicate. After 40H, media was removed from wells and cover slips were fixed in 100% methanol for 10 minutes. Immunostaining was performed by first blocking the coverslips in one mL of a 10% FBS solution in PBS for one hour. Cover slips were then incubated in a monoclonal anti-CTEB antibody at a 1:500 concentration for one hour and washed five times with a cold PBS solution. Cover slips were then incubated in an alexafluor 488 conjugated secondary antibody at a 1:1000 concentration for one hour and again washed five times with a cold PBS solution. Cover slips were then mounted onto slides using ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA) and observed under a fluorescent microscope for inclusion formation. Twenty fields of view were taken from each cover slip and triplicate cover slip counts were averaged. Averages were plotted on a graph using GraphPad Prism software and evaluated for error using standard error of the mean (SEM).

2.3 Results

2.3.1 The Presence of Mutant Tarp Diminishes Wild Type Tarp's Ability to Polymerize Actin Filaments

One of *C. trachomatis*'s key abilities to enter into a host cell is its ability to re-arrange the host cell's actin cytoskeleton thus allowing a non-phagocytic epithelial cell to uptake the bacterium. The secreted Tarp effector is a known stimulator of actin filament formation and has previously been shown to be able to polymerize actin *in vitro*. Tarp is known to have five distinct regions, a phosphorylation domain, a proline rich domain, an actin binding domain, and two F-actin binding domains (Fig. 4). Through the use of deletion mutants in previously performed pyrene assays, it has been shown that the removal of either the proline rich domain or the actin binding domain from Tarp caused a significant decrease in these deletion mutant's abilities to polymerize actin [26]. It is hypothesized that Tarp is able to function in a homo-oligomer once it enters a host cell; therefore, we speculated that a dominant

negative effect was occurring in these deletion mutants where Tarp molecules missing either their proline rich domains or actin binding domains were interfering with wild type Tarp's ability to interact with the host cell's actin cytoskeleton. To test this, purified Tarp protein from both wild type Tarp and Tarp mutants missing either the proline rich domain or actin binding domain were generated in *E. coli*, purified, and then run using SDS-PAGE to test whether they were present or not (Fig. 5A). These purified proteins were then combined in equal molar ratios and used to run a pyrene assay to test the rate of actin polymerization *in vitro* (Fig. 5B and 5C). These assays showed that when the Tarp mutants were missing either their proline rich domain or its actin binding domain, there was an observable reduction in the rate of actin polymerization when compared to actin in the presence of only WT Tarp. It was also shown that when Tarp missing its proline rich domain or actin binding domain were introduced to actin alone, there was no appreciable difference in the rate of actin polymerization between the actin only control and these mutant Tarp proteins. To ensure that the reduction of actin polymerization was a not a result of excess amounts of Tarp, both WT and mutant, present in the protein purifications, we tested purified Tarp missing its phosphorylation domain as a control. The phosphorylation domain had previously been shown to have a similar level of actin polymerization as that of WT Tarp. Both purified WT Tarp and Tarp Δphos (Fig. 5D) were mixed in equimolar ratios and the rate of actin polymerization was tested in a pyrene assay (Fig 5E). This assay showed an increase in the rate of actin polymerization when these two forms of Tarp were mixed together. This data shows that mutant Tarp can have the ability to reduce actin polymerization *in vitro* and supports our hypothesis that a dominant negative effect can occur between two forms of competing Tarp molecules.

2.3.2 *C. trachomatis* Transformants are Able to Successfully Express Mutant Tarp in Bacteria

Based on this *in vitro* data, our lab speculated that Tarp mutants which lack either the proline rich domain or actin binding domain would reduce the rate of wild type Tarp mediated actin nucleation *in*

vivo. We sought to prove this through the generation of five distinct chlamydial shuttle vectors which express a mutant form of the Tarp protein (Fig. 6A). Four of the vectors were designed to express Tarp without one of its key domains and the fifth vector was able to express WT Tarp. A region of approximately 200 nucleotides, which we have come to call the Tarp promoter, was placed in front of each of these Tarp genes to ensure their expression at the same time as the endogenous Tarp gene. Each of these shuttle vectors were then transformed into L2 *C. trachomatis* and selected for using antibiotics. We then verified that each of these transformant bacteria were able to successfully express their mutant protein through checking for the presence of their C-myc epitope tag using western blot analysis (Fig. 6B). As can be seen in pictures of the blot, Tarp is present in both our WT bacteria as well as in each of our transformants (Fig. 6B). In addition to this, the c-myc blot shows us that our transformants are producing the mutant forms of the Tarp protein due to the fact that each mutant shows a single band at the correct size (Fig. 6B). Both of these figures together show us that our transformants were able to take up the appropriate plasmid and then express a mutant Tarp protein.

2.3.3 *C. trachomatis* Transformants Expressing Mutant Tarp Exhibit a Significant Decrease in their Invasion Phenotype

One of the major requirements of the developmental cycle of *C. trachomatis* is its ability to invade a host cell which requires *C. trachomatis* to be able to nucleate actin within host cells. However, it is currently unknown whether any of the domains of Tarp have a significant effect on the ability of an elementary body to invade a host cell. Our previous *in vitro* data suggests that the removal of either the proline rich domain or actin binding domain from Tarp may have a significant effect on Tarp's ability to nucleate actin. This also suggests that the removal of these domains may have a significant effect for transformants expressing TarpΔABD or TarpΔPRD. To test the invasion phenotype of our transformants expressing mutant forms of Tarp as well as endogenous Tarp, we were able to perform an invasion assay and then observe the results of the assay under a fluorescent microscope (Fig. 7A). As expected, our

transformants which expressed both WT Tarp and TarpΔABD showed a statistically significant decrease in their ability to invade HeLa 229 host cells. Wild type *C. trachomatis* has a strong ability to invade host cells with around 79% of all bacterium placed into culture being able to be taken up into host cells within one hour of infection. The next bacterium in the figure, another WT *C. trachomatis* which expressed WT Tarp on its plasmid (pCtSV.Tarp) which has been tagged with c-myc, also showed the same rate of invasion into host cells as WT L2 *C. trachomatis*. However, the transformant missing the actin binding domain showed a decreased ability to invade host cells sitting at around 52% invasion (Fig. 7A). No altered invasion phenotype was observed for *C. trachomatis* transformants expressing either TarpΔFAB1&2 or, surprisingly, TarpΔPRD. Our most intriguing result, however, was that the expression of TarpΔPhos within a transformant resulted in a statistically significant reduction in invasion down to 34% from the 79% observed in WT L2 bacteria (Fig. 7A). This statistically significant result clearly suggests that the phosphorylation domain within Tarp has a positive effect on the ability for *C. trachomatis* to invade a host cell and also shows that when this domain is missing within our transformants, they may be capable of expressing a dominant negative phenotype. This result is surprising due to the fact that the pyrene assay performed previously suggested that the removal of the phosphorylation domain would have no effect on Tarp's ability to polymerize actin which is assumed to be a significant part of the invasion process for *C. trachomatis*.

By observing a significant change in the invasion phenotypes of both our TarpΔphos and TarpΔABD transformants, we then wanted to see if this reduction in invasion ability, assumed to be a result of a dominant-negative effect, resulted in an altered growth phenotype for our Tarp transformants. By setting up a growth curve over a 48 H period and taking time points every 12H, we were able to compare the growth phenotypes of WT L2 and all of our transformants (Fig. 7B and data not shown). As can be observed in the growth curves comparing the rates of growth of WT L2 *C. trachomatis*, *C. trachomatis* transformants expressing pCtSV.Tarp, and *C. trachomatis* transformants expressing

TarpΔPhos, there is little difference between the three in terms of either how quickly they grew or how many EBs are produced by the end of their growth curves when host cells are invaded by a normalized number of *C. trachomatis* bacterium (Fig. 7B). When looking at these results, we can conclude that while the phosphorylation domain does appear to have a negative effect on *C. trachomatis*' ability to invade a host cell, it does not appear to have any significant effect on the bacterium's ability to undergo development and expand once within a host cell.

2.3.4 *C. trachomatis* is Able to Secrete the TarpΔPhos Effector into Host Cells

In addition to confirming that our transformants are expressing mutant forms of Tarp, we also wanted to confirm that they were able to secrete these Tarp molecules into host cells. The translocation of Tarp into host cells is integral to the molecule's ability to interact with the host cell's actin cytoskeleton. Due to the significant, but unexpected, decrease in the ability for the transformant pCtSV.TarpΔphos to invade a host cell, we believed that a dominant-negative effect was occurring between both the WT and TarpΔPhos effectors being secreted into the host cell. However, it could also be viewed that the decrease in invasion was due to an inability for the bacterium to secrete Tarp into the host cell's cytosol. Our lab was able to test the transformant's ability to secrete Tarp into the host cell by performing a fractionation on a cell homogenate of host cells that were infected with our pCtSV.TarpΔphos transformant. After infecting a HeLa 229 cell monolayer and sonicating the infected cells, the homogenate was then spun down at 800xg in an ultracentrifuge and its supernatant was removed. This allowed us to collect a pellet of material and a supernatant as well. This process was then repeated for 10,000xg and 100,000xg. Each of these pellets was then resuspended in protein buffer and the final soluble fraction from the 100,000xg spin down was kept back. Each pellet and the soluble fraction was then run on a 10% polyacrylamide gel. The gel was then transferred to a nitrocellulose membrane and probed with an α Tarp, α c-myc, α actin, α EB, α MOMP, or α GAPDH antibody. As can be seen in the α EB blot, EBs are completely taken out of

the homogenate by the end of the 10,000xg spin down however both the α Tarp and α c-myc blot both show that not only WT Tarp but also our mutant form of Tarp missing the phosphorylation domain are present in the soluble fraction as well as the 100,000 x g pellet, which represents the host cell cytosolic fraction as defined by the presence of the soluble eukaryotic protein GAPDH. (Fig. 8B). In addition, we were able to perform a temperature controlled experiment where neither endogenous nor mutant Tarp were detected in host cells that were incubated with *C.* trachomatis EBs at 4^{o}C. It has been shown in previous experiments that secretion of effectors through the type III secretion system in *C. trachomatis* is temperature dependent. This control has the benefit of showing that no Tarp is present in the 100,000xg pellet or soluble fraction of *C. trachomatis* bacterium that cannot use their type III secretion systems. By looking at the results from this figure, we can conclude that the *C. trachomatis* transformants that we have created are both successfully able to produce mutant forms of Tarp and then are able to secrete those Tarp molecules directly into the host cell's cytosol.

2.3.5 TarpΔPhos Expressed on a Plasmid is Able to Co-Immunoprecipitate with WT Tarp

The oligomerization of multiple Tarp proteins once these proteins are secreted into the host cell's cytosol is thought to be an integral part of how Tarp is able to polymerize actin and form new actin filaments within the host cell. We hypothesize that the necessary formation of these oligomers causes the dominant-negative effect observed when WT Tarp and TarpΔPhos are secreted together. However, it is unknown if these two proteins are able to form a complex together. Our group decided that by immunoprecipitating the two proteins together, then this would show that they are able to form a complex and that they may be causing the dominant-negative effect within our transformant. A T175 monolayer of McCoy cells were infected with pCtSV.Tarp Δphos transformants for two hours and were then homogenized using a sonicator. This homogenate was then fractionated out to 10,000xg where the supernatant was then run through a column containing anti c-myc sepharose beads. The beads were then

collected, dissolved in protein sample buffer, and run using SDS-PAGE. After transferring the proteins to a nitrocellulose membrane, the homogenate sample was tested for the presence of both Tarp, using an anti-Tarp polyclonal antibody, and actin, using an anti-actin polyclonal antibody. In the Tarp blot, two separate bands were observed: one at ~150 kiloDaltons (kDa) and one at ~100 kDa. The Band at 150 kDa matches with the expected size for WT Tarp and the band at 100 kDa matches with the expected size for Tarp with the phosphorylation domain removed (Fig. 9). Since the beads used to purify the Tarp protein from the rest of the homogenate targeted the c-myc tag, which is only found on the mutant forms of tarp, we can safely assume that both the mutant Δphos Tarp and WT Tarp are able to interact with each other and form a complex. This is further supported by the fact that actin was found in the same homogenate. Since Tarp is normally able to bind to actin as a part of forming a complex, the presence of this band shows that a true complex between TarpΔPhos, WT Tarp, and actin had been formed *in vivo* (Fig. 9). This figure shows us that WT Tarp has the ability to form a complex with a mutant form of Tarp and lends further evidence to the possibility that a dominant-negative effect may be causing the invasion deficiency in our transformants.

2.3.6 Removal of the Proline Rich Domain from TarpΔphos is Able to Restore its Invasion Phenotype to WT Levels

After showing that a complex is able to be formed between a mutant form of Tarp and WT Tarp, the final question that arose was whether the original WT invasion phenotype could be restored in our TarpΔphos transformant. Our lab came to believe that by removing the proline rich domain from the TarpΔphos mutant protein, it would remove the protein's ability to oligomerize with WT Tarp and would thus restore the WT invasion phenotype in the transformant. By using the PCtSV.Δphos plasmid and then removing the proline rich domain from it, our lab was able to create a new plasmid. This plasmid was then transformed into *C. trachomatis* thus allowing us to generate a transformant that expressed both WT Tarp and Δphos ΔPRD Tarp (Fig. 6A). Upon performing an invasion assay using this new transformant, it

was observed that the invasion phenotype was restored to WT levels of invasion sitting at around ~80% of all *C. trachomatis* placed in the culture invading the host cell (Fig. 10B). In addition to this, there was no observed defect in the transformant's ability to develop within host cells (data not shown).

2.4 Discussion

Chlamydia trachomatis is an obligate intracellular parasite that contains multiple early effectors that allow it to invade an epithelial host cell. One of the most well-known of these factors is the Translocated Actin Recruiting Phosphoprotein (Tarp). Tarp is speculated to play a large role in triggering the process of entry into a host cell through stimulation of actin polymerization and formation of an actin pedestal that the *C. trachomatis* EB can attach itself to. Through the use of a chlamydial transformation system, our lab was able to express mutant Tarp proteins that are missing one of the five key domains of Tarp including the phosphorylation domain, proline rich domain, actin binding domain, and f-actin binding domains 1 and 2. These transformants were then tested to see if they caused a deficiency in invasion and/or growth and development within a live host cell. As hypothesized, *C. trachomatis* transformants expressing a Tarp effector without the actin binding domain alongside genomic Tarp showed a deficiency in their ability to invade host cells. This result mirrors previously established results from *in vitro* pyrene assays which showed that the presence of Tarp missing the actin binding domain reduced the potential for actin nucleation. Conversely, those transformants that expressed Tarp missing the proline rich domain did not show any reduction in their ability to invade a host cell. Given previous *in vitro* pyrene assay data suggesting that the removal of this domain from Tarp and expression alongside WT Tarp caused a decrease in actin nucleation, this result is somewhat surprising. However, upon further review the results found within the pyrene assay may be an artifact of the *in vitro* experiment. The presence of TarpΔPRD has been shown to be able to sequester monomeric actin in a concentration dependent manner in previous *in vitro* experiments and would reduce the amount of monomeric actin

available in the assay [35]. *In vivo*, the major effect that the removal of the proline rich domain from TarpΔPRD has is that the mutant proteins lack the ability to oligomerize with endogenous WT Tarp. Due to the results observed in the invasion assay for the proline rich domain transformant, it would appear that neither inability to form a complex with endogenous Tarp nor the sequestration of monomeric actin has a significant effect on the transformant's ability to invade a host cell. While the removal of either the proline rich domain or the actin binding domain from Tarp was predicted to have an effect on the ability for *C. trachomatis* to invade a host cell, the removal of F-actin binding domains 1&2 from the Tarp protein was not predicted to have any effect on the transformant's ability to be taken up into a host cell. This result was confirmed in the invasion assay.

The most interesting result from our experiment was that the removal of the phosphorylation domain from a Tarp deletion mutant and expressing it alongside genomic WT Tarp caused the largest reduction in rates of invasion. This was surprising due to the fact that, in the past, the inhibition of the Tarp phosphorylation domain through the use of PP2 inhibitors did not show any reduction in rates of invasion. Based on previous *in vitro* pyrene assays, we cannot assume that this reduction in invasion rates is due to the disruption of the direct actin nucleation activity of endogenous Tarp. Instead, it is more likely that a dominant-negative effect is occurring due to the formation of a heterocomplex between these two proteins once they both are secreted into the host cell's cytosol. This thought is further assured when, through the use of co-immunoprecipitation, our lab was able to observe that endogenous WT Tarp and TarpΔphos are able to form a heterocomplex with one another. Finally, we were able to remove the proline rich domain from the TarpΔphos mutant protein and then secrete this altered Tarp into a host cell alongside genomic Tarp only to observe that WT levels of invasion were restored. From these results, it may be easy to assume that the phosphorylation domain plays a key role within the invasion of *C. trachomatis* into a host cell; however, previous studies have established that phosphorylated Tarp still requires other host factors to activate actin nucleation. One key difference between previous studies and

this study is that, in previous studies, the phosphorylation domain of Tarp was merely inhibited and in this study, the phosphorylation domain was completely removed. This leaves the assumption that the phosphorylation domain of Tarp is necessary for invasion to be an assumption that cannot be fully supported at this time. Before a true conclusion can be made, more mechanistic data about Tarp and its role in the invasion of a host cell must be acquired.

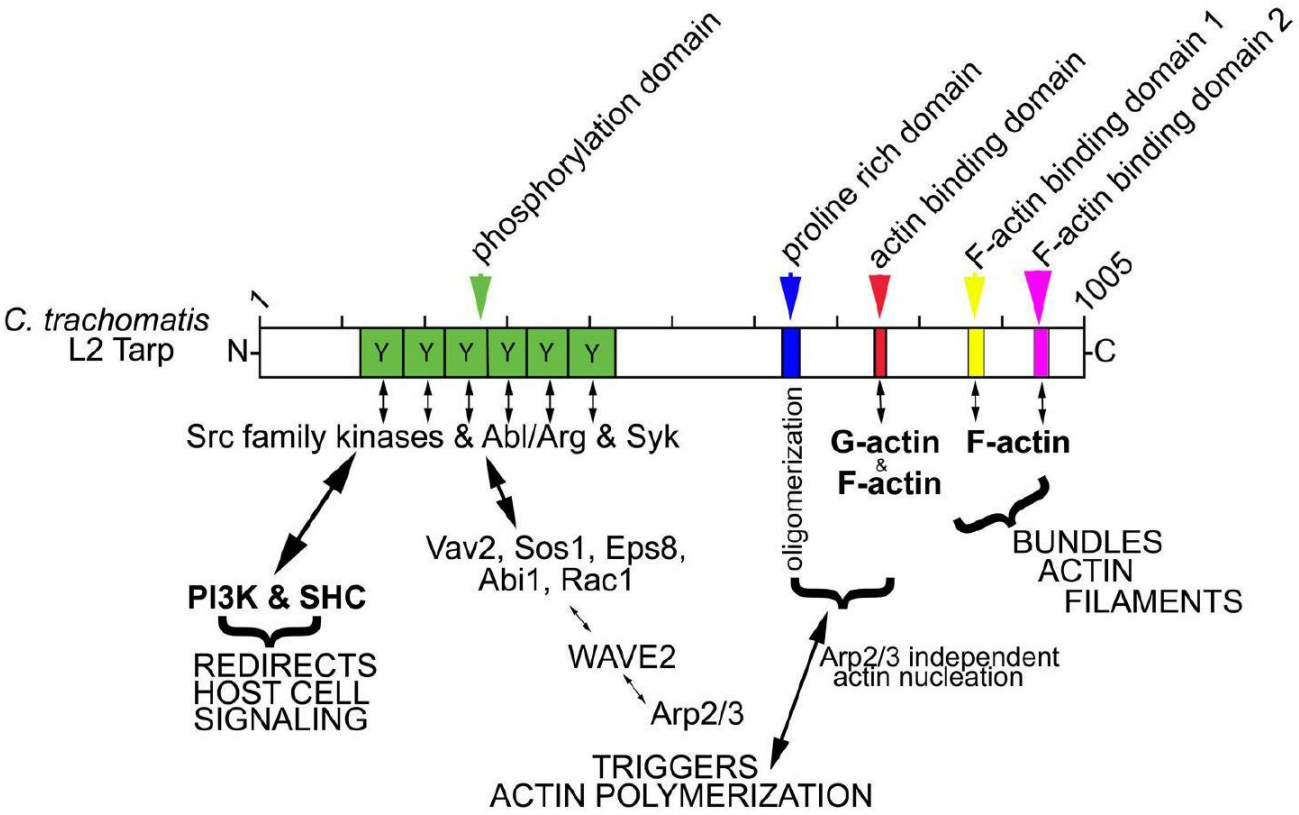

Figure 4 The Tarp protein and the signaling pathways it utilizes to enter a host cell

C. trachomatis Tarp is comprised of five primary regions. The first region is the tyrosine rich repeat phosphorylation domain located closest to the N-terminus. This region is phosphorylated by Src family kinases hijacked from the host cell including Src, Yes and Fyn as well as by other tyrosine kinases such as Syk or Abl/Arg kinases. Once Tarp is phosphorylated by these host cell kinases, it is also thought to be able to associate with the host cell Src homology 2 domain containing protein 1 (SHC1) and the phosphoinositide 3-kinase (PI3K) which allows Tarp to create a protective niche for itself within the host cell due to the resulting changes in the way that host cell signals are activated. The next region within the Tarp molecule, the proline rich domain, is thought to allow the Tarp molecule to oligomerize while the third domain within the molecule, the actin binding domain, is thought to allow Tarp to bind to both G- and F-actin. Both of these domains together are implicated in an Arp2/3 independent pathway that allows Tarp to nucleate new actin filaments. Finally, the last two domains within Tarp, F-actin binding domains 1&2, are thought to aid Tarp in the bundling of existing actin filaments along with the previously mentioned actin binding domain.

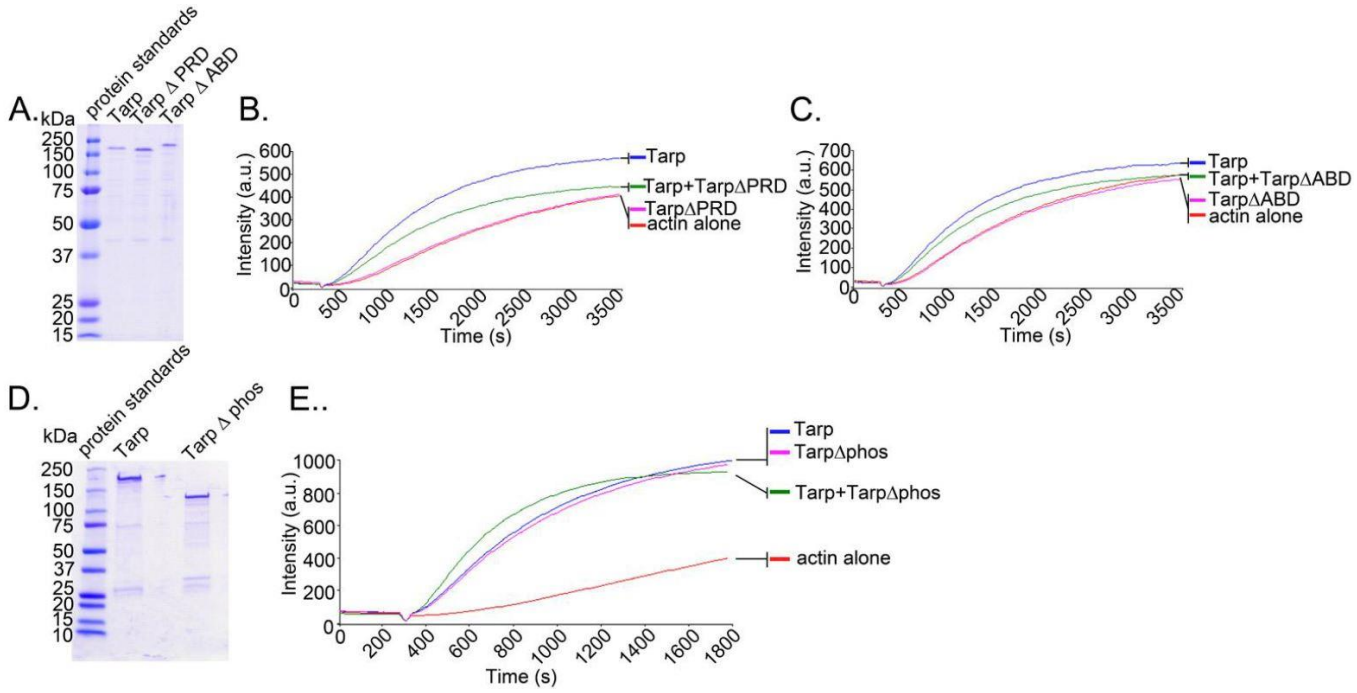

Figure 5 Tarp mutants inhibit the ability for actin to be nucleated *in vitro*

(A) Purified WT Tarp and Tarp mutants with deletions of the proline rich domain and actin binding domain were resolved by SDS-PAGE and then stained using Coomassie blue. (B) The Tarp ΔPRD deletion mutant was observed to inhibit WT Tarp mediated actin nucleation in pyrene actin nucleation assays due to the observed decrease in the slope of the pyrene assay. Equal concentrations of both WT Tarp and Tarp ΔPRD proteins were incubated with 1μM monomeric pyrene-labeled actin. An increase in actin polymerization after the addition of polymerization buffer at 300 seconds was measured as arbitrary fluorescence intensity (Intensity (a.u.)) over time (Time(s)). Pyrene actin alone served as a negative control. (C) The Tarp ΔABD deletion mutant was also observed to interfere with WT Tarp mediated actin nucleation in pyrene actin nucleation assays. The experiment was designed as described in B using Tarp ΔABD instead of Tarp ΔPRD. (D) Purified WT Tarp and a Tarp mutant harboring a deletion in the tyrosine rich phosphorylation domain (Δphos) were resolved by SDS-PAGE and visualized by Coomassie blue staining. (E) The Tarp Δphos deletion mutant enhances wild type Tarp mediated actin nucleation in a pyrene actin nucleation assay as an increase in the slope in the pyrene actin assay was observed. The pyrene curve generated by Tarp Δphos and wild type Tarp was equivalent to a 2x concentration of wild type Tarp curve (data not shown). Experiment was performed similar to panels B & C.

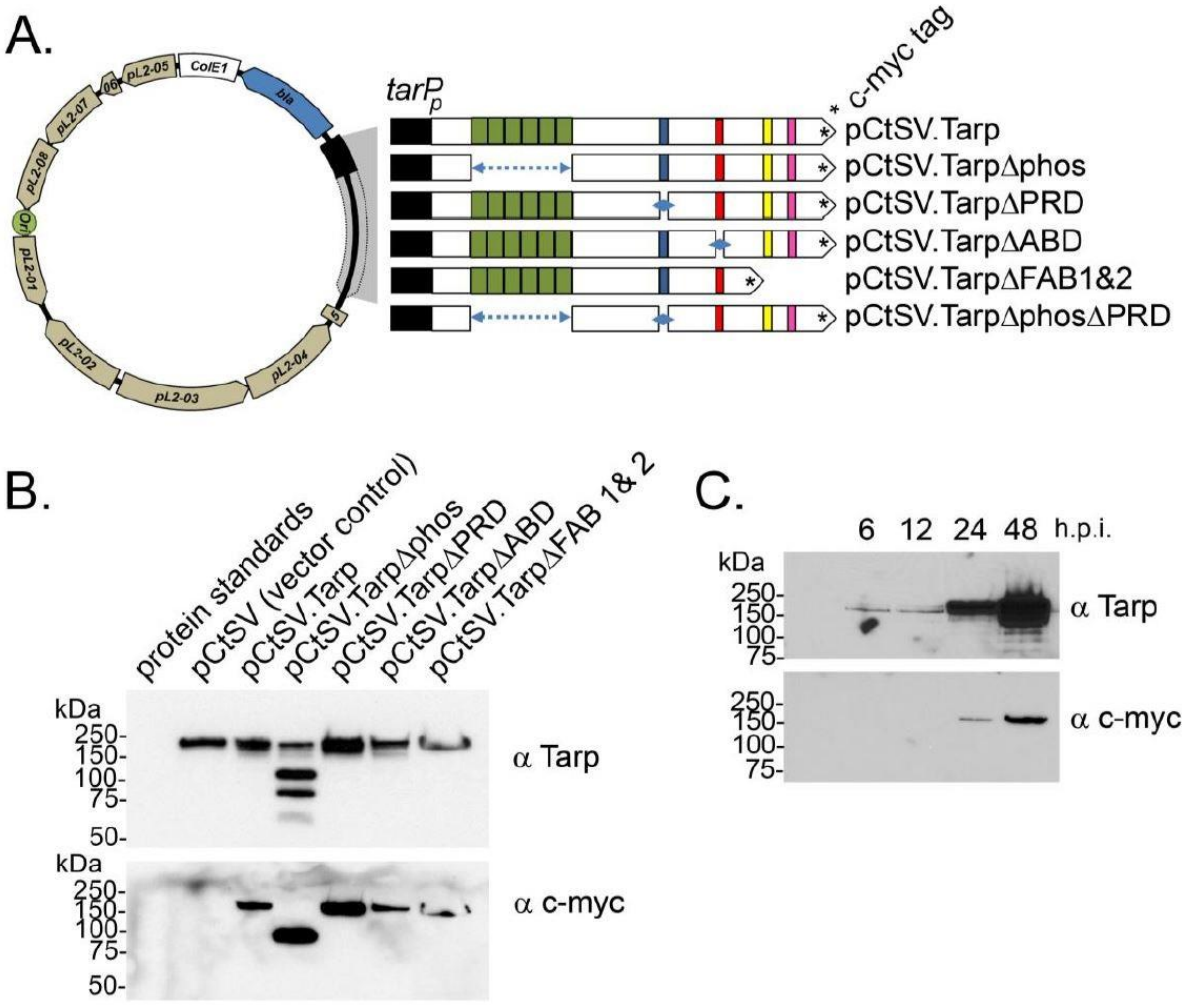

Figure 6 The shuttle vector PctSV.1 and the mutant Tarp proteins that it can express

(A) The *C. trachomatis* shuttle vector pCtSV.1 was adapted to allow for the expression of c-myc tagged Tarp under the control of the *tarP* promoter (*tarPp*). In frame deletions were generated in the *tarP* gene to remove the phosphorylation domain (pCtSV.Tarp Δphos), proline rich domain (pCtSV.Tarp ΔPRD), g-actin binding domain (pCtSV.Tarp ΔABD) f-actin binding domains (pCtSV.Tarp ΔFAB1&2), and the double deletion mutant, a phosphorylation domain and proline rich domain mutant (pCtSV.Tarp Δphos ΔPRD). (B) Protein lysates were generated from McCoy cells infected with *C. trachomatis* L2 that had been transformed with one shuttle vector including pCtSV.1, pCtSVTarp, pCtSV.Tarp Δphos, pCtSV.TarpΔPRD, pCtSV.Tarp ΔABD, and pCtSV.Tarp ΔFAB1&2. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis with Tarp (α Tarp) and c-Myc (α c-myc) specific antibodies. Molecular mass is in kDa. (C) *Chlamydia trachomatis* (+pCtSV.Tarp) Infected host cells were collected from a 6 well plate at 6, 12, 24 and 48 hours post infection and solubilized in protein sample buffer. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis with Tarp (α Tarp) and c-Myc (α c-myc) specific antibodies.

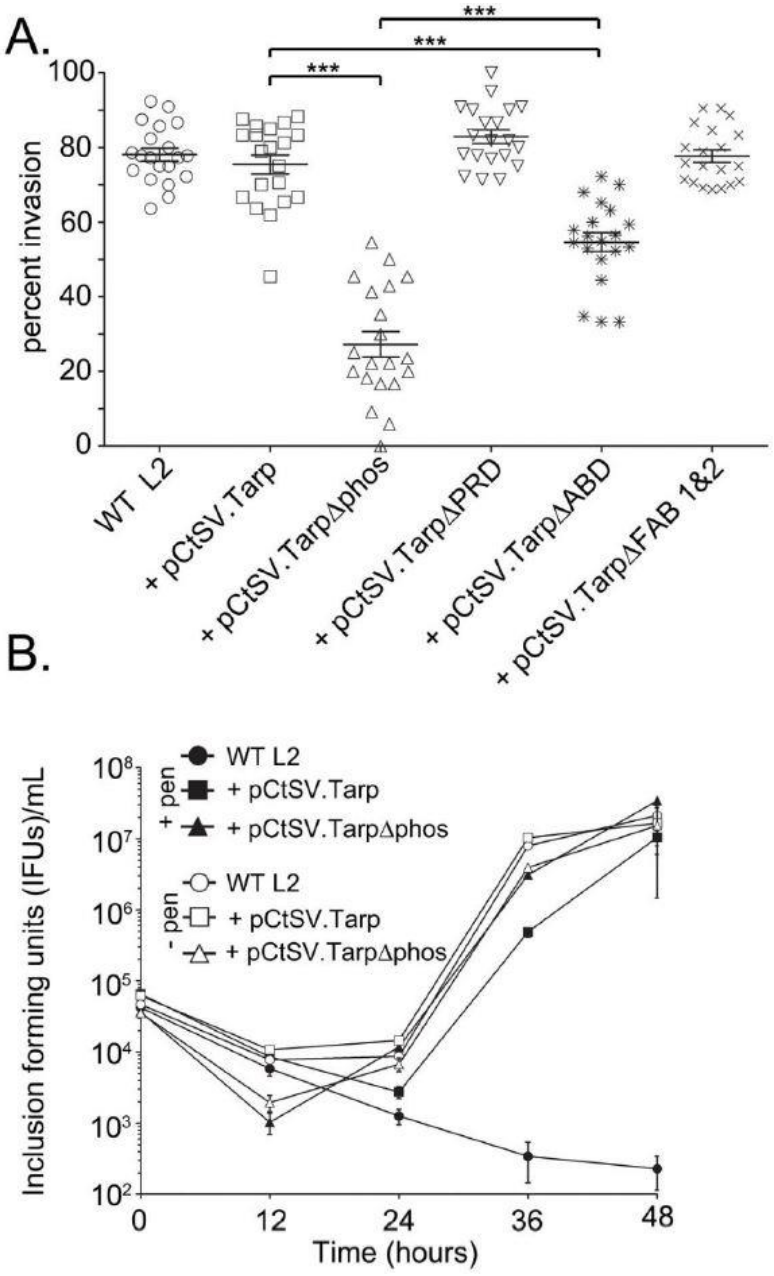

Figure 7 Invasion and growth of *C. trachomatis* transformants *in vivo*

(A) Wild type *Chlamydia trachomatis* (L2; circles) or L2 transformants harboring plasmid pCtSV.Tarp (+ pCtSV.Tarp; squares), pCtSV.TarpΔphos (+ pCtSV.TarpΔphos; triangles), pCtSV.TarpΔPRD (+ pCtSV.TarpΔPRD; inverted triangles), pCtSV.TarpΔABD (+ pCtSV.TarpΔABD; asterisks), or pCtSV.TarpΔ FAB1&2 (+ pCtSV.TarpΔFAB1&2; "x"), were examined for chlamydial invasion of HeLa 229 cells. All EBs used in invasion assays were labeled using the red fluorescent cell tracker dye CMPTX. After allowing 1 hour for invasion, extracellular EBs were counterstained by indirect

immunofluorescence with a monoclonal antibody to *C. trachomatis* L2 MOMP and a goat anti mouse antibody conjugated to Alexa 488. The data are represented as the percentage of intracellular EBs relative to the total number of extracellular and intracellular EBs per field of view. Each data point represents a single field of view at 1000X magnification. Data sets were compared with one way ANOVA and Tukey's multiple comparison test of the mean. *** represents a p value of < 0.001. (B) The development of wild type *C. trachomatis* L2 (circles) and transformants harboring plasmid pCtSV.Tarp (+ pCtSV.Tarp; squares), pCtSV.TarpΔphos (+ pCtSV.TarpΔphos; triangles) were observed over a 48 hour period, after normalizing the initial multiplicity of infection for each clone. Infected cells with antibiotic selection (black shapes) and infected cells without antibiotic selection (open shapes) were collected at t=0, 12, 24, 36 and 48 hours post infection and mechanically lysed using sonication to release and then collect infectious EBs. IFUs were determined for each transformant by serial dilution of released EBs harvested at each time point and reinfection of HeLa cells grown on coverslips to determine the number of IFUs per mL of harvested material.

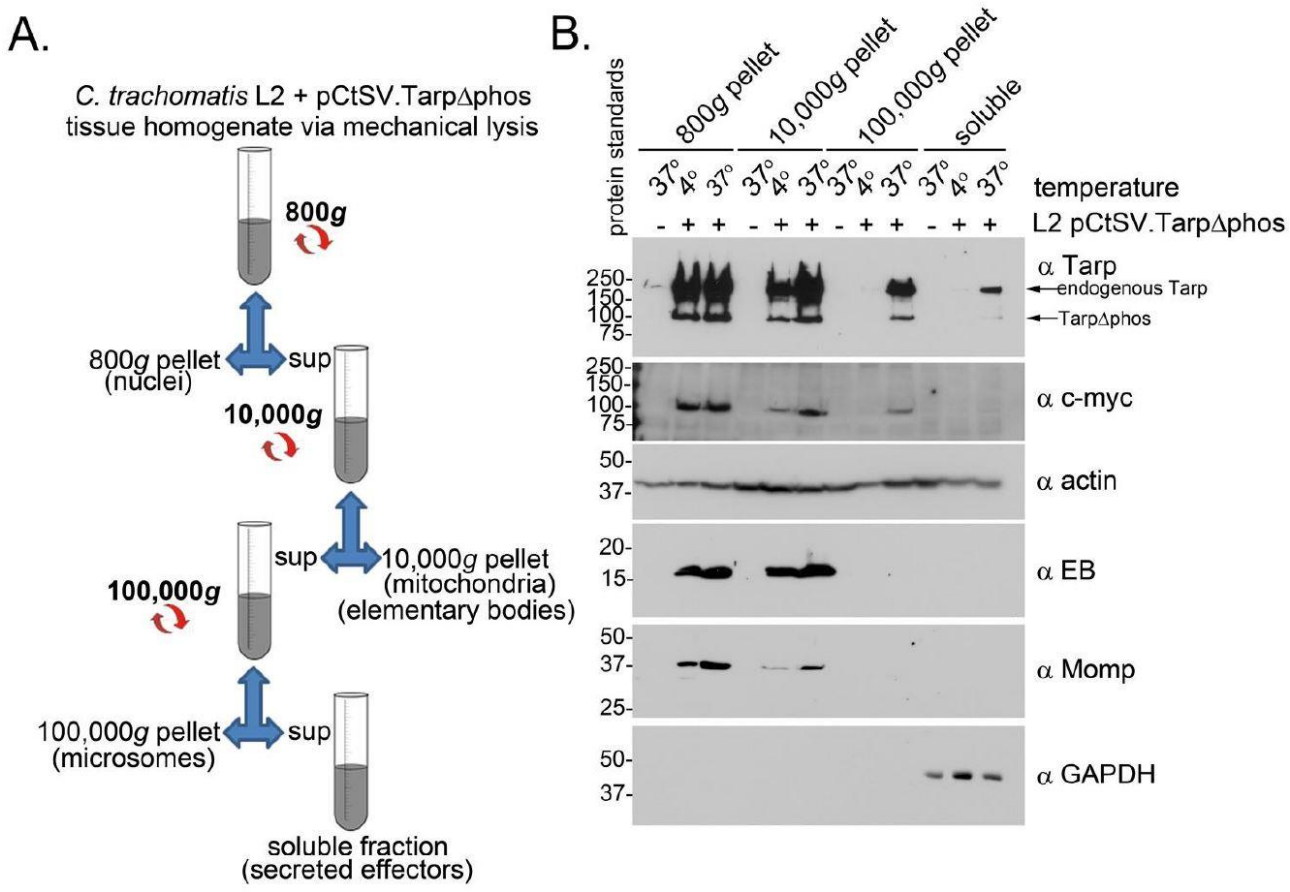

Figure 8 *C. trachomatis* is capable of expressing and secreting both mutant Tarp and genomic Tarp

(A) A representative schematic of the process of fractionation used to collect the 800xg, 10,000xg, 100,000xg pellets and soluble fraction used to run the western blot in panel B. Sonication was used to break open infected host cells and create the original tissue homogenate. (B) Starting in the 100,000xg pellet, a soluble Tarp fraction can begin to be observed without the presence of EBs within host cells infected with the *C. trachomatis* serovar L2 transformed with the shuttle vector pCtSV.TarpΔphos (L2 pCtSV.TarpΔphos). This observation can also be seen within the soluble fraction of the tissue homogenate. Fractions were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblot analysis with antibodies specific for Tarp (α Tarp), c-myc epitope (α c-myc), elementary bodies (α EB), *C. trachomatis* major outer membrane protein (α Momp), Glyceraldehyde 3-phosphate dehydrogenase a soluble protein marker (α GAPDH) and actin a protein expected to be present in all fractions (α actin).

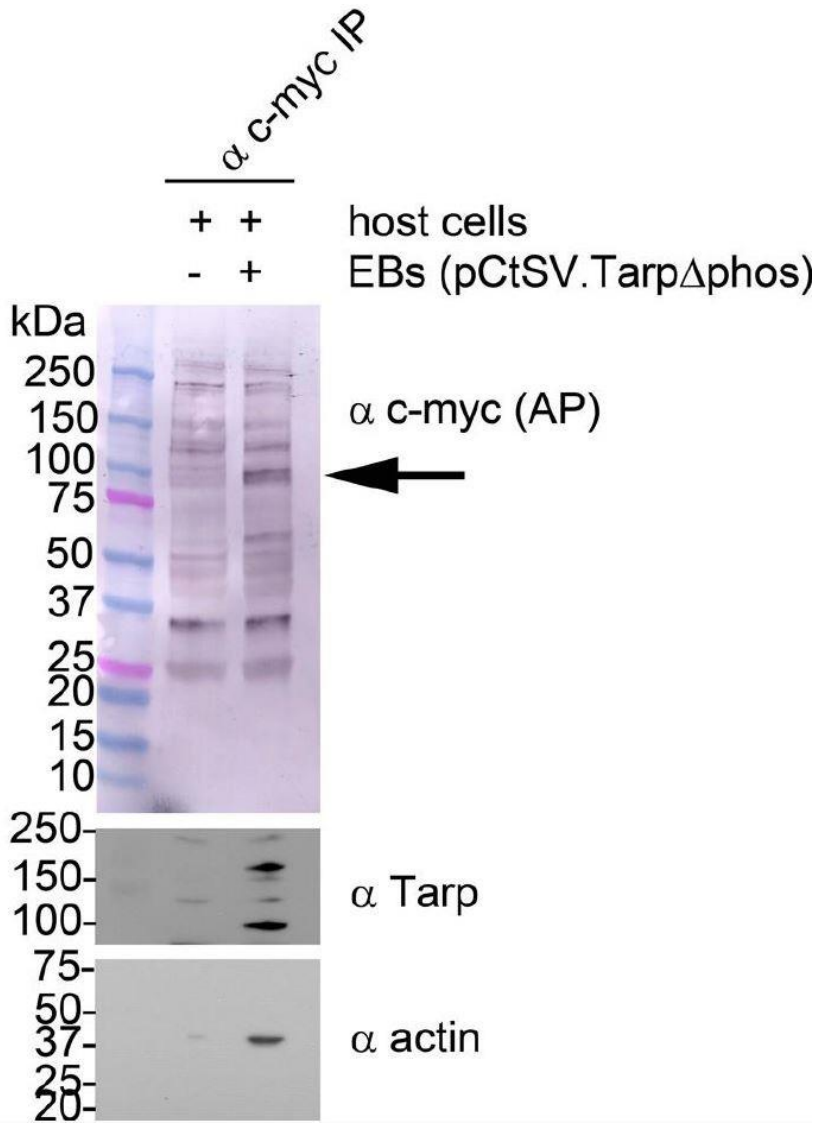

Figure 9 Genomic Tarp is capable of forming a complex with TarpΔphos once both are transferred into the cytosol of a host cell

By incubating a 10,000xg pellet sample with agarose beads with an alkaline phosphatase conjugated c-myc antibody, we were able to observe that mutant Tarp and WT Tarp from pCtSV.TarpΔphos transformants were able to be immunoprecipitated together. As described in figure 8a, two 10,000xg pellets were created from either an empty T175 flask of McCoy cells or a T175 flask of McCoy cells infected with *C. trachomatis* pCtSV.TarpΔphos transformants. These pellets were then dissolved in protein sample buffer and Proteins were resolved by SDS-PAGE and immunoblotted with Tarp (α Tarp) and actin (α actin) specific antibodies. Immunoprecipitation of a c-myc tagged protein of the correct molecular weight for TarpΔphos (arrowhead) was observed directly on the nitrocellulose membrane following an incubation with an alkaline phosphatase conjugated c-myc antibody (α c-myc AP) and corresponding substrate.

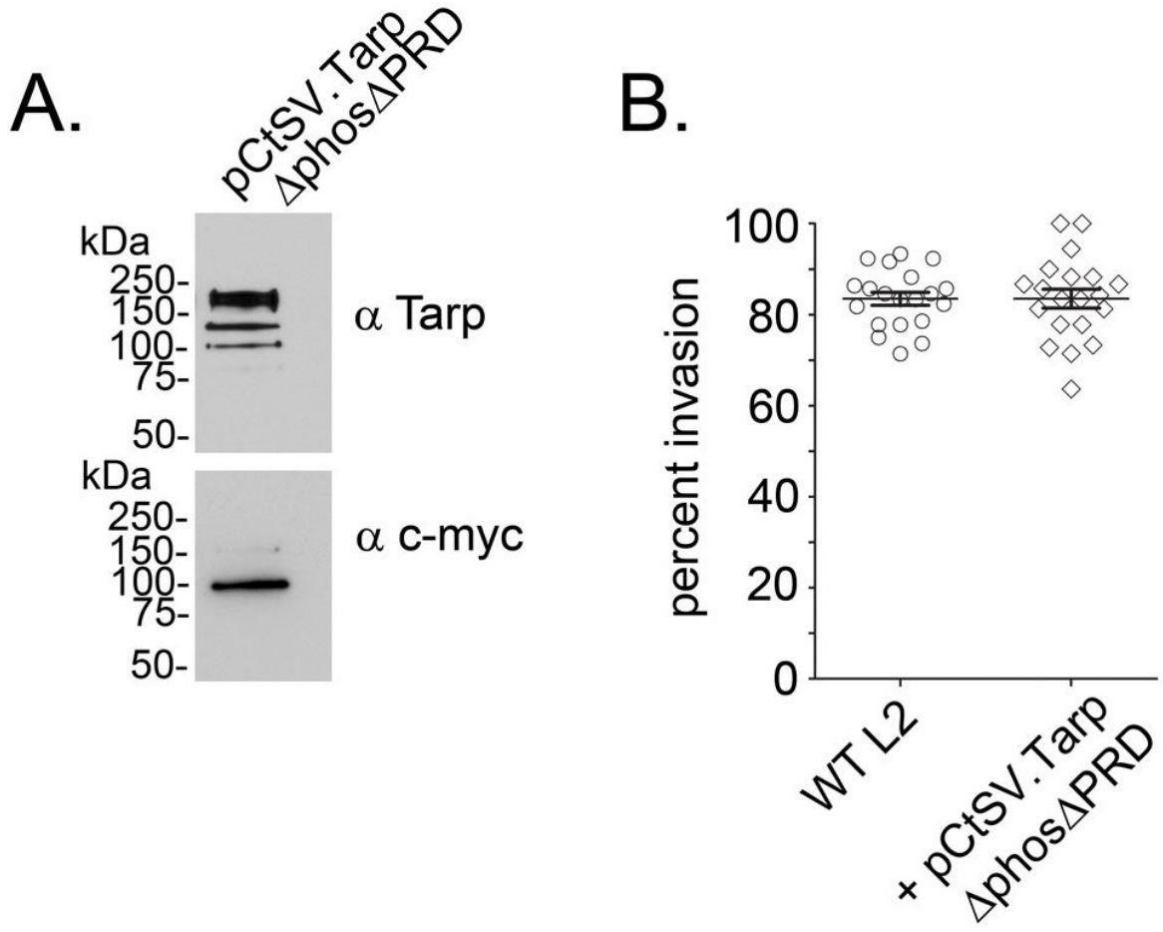

Figure 10 Removal of the proline rich domain from TarpΔphos transformants restores the invasion phenotype to WT levels

(A) A double mutant Tarp protein missing both its phosphorylation domain and proline rich domain by deleting the proline rich domain from the pCtSV.TarpΔphos shuttle vector creating the shuttle vector pCtSV.TarpΔphosΔPRD. This vector was then transformed into WT L2 C. trachomatis and used to infect McCoy cells. The double mutant protein was then purified from these infected host cells and resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis with Tarp (α Tarp) and c-myc (α c-myc) specific antibodies. Molecular mass is in kDa. (B) As described previously, C. trachomatis transformants were examined for bacterial invasion of host cells. EBs were examined for chlamydial invasion of HeLa 229 cells. All EBs used in invasion assays were labeled using the red fluorescent cell tracker dye CMPTX. After allowing 1 hour for invasion at 37^{o}C, extracellular EBs were counterstained by indirect immunofluorescence with a monoclonal antibody to C. trachomatis L2 MOMP and a goat anti mouse antibody conjugated to Alexa 488. The data are represented as the percentage of intracellular EBs relative to the total number of extracellular and intracellular EBs per field of view. Each data point represents a single field of view at 1000X magnification.

CHAPTER 3: CONCLUSION

3.1 Discussion of Data

Chlamydia trachomatis is one of three species within the genus *Chlamydia* that is able to infect humans. This organism relies on its ability to invade host cells so that it can replicate within an inclusion through binary fission [53]. For this organism to be able to achieve its goal of invading a host cell, it has a set of effector proteins that are stored inside of its elementary body (EB) form that are injected directly into the host cell's cytosol using a type III secretion system. There are three known effectors stored within the *C. trachomatis* EB, but the effector that is by far the most well understood in both form and function is the Translocated actin recruiting phosphoprotein (Tarp) effector [32]. The Tarp effector is thought to promote invasion of the EB into the host cell through actin cytoskeleton rearrangements and the bundling of actin filaments. Tarp is known to be present in most forms of chlamydia. Previous *in vitro* experimentation has established Tarp's ability to interact with host factors such as actin; however, the ability of this protein to interact with host factors *in vivo* and whether Tarp plays a role in *Chlamydia*'s ability to invade or grow inside of a host cell is unknown. This research provides the first *in vivo* Tarp data using transformants that express mutant Tarp alongside endogenous Wild Type (WT) Tarp. In contrast to previous *in vitro* research, this data suggests that key regions of the Tarp protein may play a significant role in the invasion process and that the phosphorylation domain of Tarp may be involved in the invasion of *C. trachomatis* into a host cell. This research also confirms previous *in vitro* data about the Actin Binding Domain (ABD) and shows that it may play a role in invasion *in vivo.*

One of the five major regions within the *C. trachomatis* Tarp molecule is the Actin Binding Domain. This domain has been identified through previous *in vitro* studies as being able to bind to both G- and F-actin. These same studies have presumed it as a key region, along with the Proline Rich Domain of Tarp, that allows *C. trachomatis* to initiate the nucleation of actin [26]. In addition to these previous

discoveries, it is also known, through *in vitro* pyrene assays done in previous studies, that the deletion of the Actin Binding Domain from Tarp causes a decrease in the levels of actin nucleation when compared to using purely WT Tarp [34]. With this previous foundation of knowledge in place, we were then able to assume that the Actin Binding Domain would be a region that would have a great effect on the ability for *C. trachomatis* to invade a host cell if ever altered *in vivo*. This expected result is confirmed in our invasion assays. By removing the Actin Binding Domain from Tarp, those *C. trachomatis* transformants showed a statistically significant decrease in their invasion phenotype, from about 80% to 50%, when compared to WT *C. trachomatis*. This decrease in invasion percentage allows us to stipulate that the Actin Binding Domain of Tarp may play a larger role in the ability for *C. trachomatis* to invade a host cell.

The Phosphorylation domain is considered to be another key domain of the Tarp molecule within *C. trachomatis*. This N-terminus domain is unique to *C. trachomatis* as it is not found within the Tarp molecule within any other *Chlamydia* species and is tyrosine phosphorylated upon entry into a host cell. In a similar vein to the Actin Binding Domain, the removal of the Phosphorylation domain from Tarp proved to have a statistically significant decrease in the invasion phenotype, from about 80% to 30%, of those *C. trachomatis* transformants. The removal of the Phosphorylation domain causing such a drastic reduction in the invasion phenotype was not something that was predicted upon starting this experiment. According to previous studies, the inhibition of host tyrosine kinases through the use of the tyrosine kinase inhibitor PP2 did not cause a significant change in the invasion phenotype of L2 *C. trachomatis* [33]; however, this experiment was done using WT Tarp. This observed data allows us to hypothesize that the phosphorylation domain may play a larger role in the invasion of a host cell than originally thought.

In both of these cases, it should be noted that these changes are only due to these transformants producing mutant proteins alongside the WT Tarp protein. The original genomic Tarp has not been

altered in any way. This suggests that the interference in both *C. trachomatis* transformants may not be entirely due to mutant proteins being expressed. It may, in fact, be due to the interactions that the mutant proteins have with WT genomic Tarp. Tarp is normally able to oligomerize to be able to concentrate multiple proteins in one area so that it can stimulate actin polymerization. The Proline Rich Domain is responsible for Tarp's ability to oligomerize [34] and may be the domain responsible for why these decreased invasion rates are occurring. Due to the fact that these transformants are producing two separate proteins, it is possible for three separate types of oligomerization configurations to form: an oligomer of all WT proteins, an oligomer of all mutant proteins, or an oligomer of both WT and mutant proteins. It can be assumed that the oligo of all WT proteins would not be responsible for any deficiency in invasion due to WT L2 *C. trachomatis* showing a normal phenotype for invasion. In the next scenario, the mutant proteins are only interacting with one another and could show an altered phenotype due to the lack of a key region. In the final case, a unique scenario is presented to us in that we are only sure that this interaction is capable of occurring but not the effects that this interaction may have on the WT protein involved in the oligomer. More mechanistic data would be required to extrapolate a true model but it can be hypothesized that a dominant-negative effect is occurring where the mutant protein is interfering with the WT protein's ability to allow for proper interaction with factors within the host cell to promote invasion. This hypothesis is further vetted by the fact that, upon removal of the proline rich domain from mutant Tarp without a Phosphorylation domain, the invasion phenotype was restored to its WT levels.

3.2 Future Directions

Ultimately, what this research presents is the successful use of a shuttle vector to allow production of a mutant protein *in vivo*; however, while the results we have observed are incredibly interesting due to the fact that it both shows the first *in vivo* data analyzing Tarp and draws interest to a region of Tarp previously thought to play no role in invasion, these results are mostly seen as a stepping

stone on the path to the generation of true Tarp mutants. A key set of future experiments that will have to be performed will be the use of these shuttle vectors expressing mutant Tarp proteins to create knock out versions of the L2 serovar *C. trachomatis* bacteria. One method that can be used to delete Tarp would be the TargeTron ™ system. The TargeTron ™ system uses transposon mutagenesis to insert a group II intron directly into a specific sequence of DNA which allows for direct disruption of a target gene. Through the use of three primers, a specific intron sequence can be targeted within the gene of interest and a 1-step assembly PCR can be run to mutate the targeted intron. This 350 bp mutation can then be cloned into the TargeTron ™ intron expression vector which is then transformed into a host cell and used to express a RNA-protein complex (RNP). This RNP will target the gene of interest within the bacterial host cell and insert its RNA directly into the sequence. Finally, the RNA will be transcribed into cDNA and then any breaks will be repaired to create a permanent intron to disrupt the gene of interest. An antibiotic resistance marker will normally be inserted into the chromosome alongside the intron to allow for selection of the bacteria that have the gene of interest disrupted. Normally, this antibiotic is kanamycin but, for our purposes, we would use penicillin or chloramphenicol. This system has been successfully used to disrupt gene expression in multiple bacteria including *Escherichia coli*, *Lactococcus lactis*, *Clostridium perfringens*, and *Staphylococcus aureus* [54, 55, 56]. It has also been used to successfully disrupt the expression of the nonessential *incA* gene in *C. trachomatis* [57]. Given this system's widespread use and success in disrupting one other *C. trachomatis* gene, it is not beyond reason to assume that this same system will allow us to disrupt the expression of WT Tarp, allowing us to create true mutants instead of just transformants. As of now, we cannot be sure if the invasion deficiency phenotypes that have been observed are due to a dominant negative phenotype or if the removal of these domains is truly responsible for the deficiency. By removing the ability for WT Tarp to be produced, we will be able to see if an invasion deficiency will continue to occur in both the Actin Binding domain and Phosphorylation domain mutants, possibly even extending the deficiency into an outright inability to

invade host cells. In addition, we will be able to re-test whether or not the other domains, the Proline Rich Domain and the two F-Actin Binding Domains (FAB) on the C-terminus of the protein, have any significant effect on the bacterium's ability to invade host cells. However, the use of the shuttle vector to create a true knock out also has certain implications about the Tarp molecule. Mainly, it will show us if the Tarp molecule is essential or not to *C. trachomatis'* ability to invade a host cell and, thus, can be considered an essential gene. When a true knock out is created that removes either one of the five key regions of Tarp or the Tarp molecule itself, it will first be observed whether or not this mutant is able to undergo a successful developmental cycle or even attach to and invade host cells. If it cannot undergo these specific functions, then it can be determined that either that region of Tarp or the Tarp protein itself is an essential gene and cannot be studied through its removal. Instead, the Tarp gene will have to continue to be studied *in vitro* and *in vivo* using the shuttle vector to express mutant forms of the protein.

Even if Tarp proves to be essential to the survival of *C. trachomatis*, the successful use of this shuttle vector to express a mutant protein that is successfully integrated into the EB form of the bacterium can have other essential uses. Namely, it can be used to generate knock outs or even express mutant forms of other proteins within the bacterium to see if any other proteins are essential or play a greater role in the survival, growth, or invasion of the bacterium. One particular set of interesting knockouts to generate would be to remove another Type III secreted effector, such as CT166 or CT694, from *C. trachomatis* and see if this generated a deficiency in invasion and/or growth of *C. trachomatis* This would allow us to begin to characterize these other two secreted effectors and begin to determine whether or not they play an active role in the pathogenicity of *C. trachomatis*. This system that was developed also can be used to test more than what the removal of Type III secreted effectors does to the ability of the bacterium to invade/develop within a host cell. For example, it would be possible to remove certain variable domains of Major Outer Membrane Proteins (MOMPs) or to remove its ability to produce the MOMP entirely using this technology. The MOMP of *C. trachomatis* is currently identified to be an adhesin that has the

ability to bind to the heparin sulfate domains on the surface of a potential host cell allowing for invasion of said host cell [58, 59]. It was shown that through the blocking of variable domains two and four through the use of antibodies that invasion of a host cell could be completely inhibited in hamster kidney cells [59]. It would be very interesting to repeat this experiment and remove the same domains from the MOMP gene to see if the results of this study are mirrored.

Assuming that Tarp can be altered or removed without causing *C. trachomatis* to be unable to initiate its reproductive cycle, it would also be very useful to be able to expand our knowledge about Tarp outside of the L2 serovar of *C. trachomatis*. Using the shuttle vector to create knock outs of both the important regions of Tarp as well as the Tarp molecule itself in the other serovars of *C. trachomatis* (A – L1, L3) and then testing these mutants to see if they would behave the same in both invasion and development phenotype as the L2 knockout could be very useful as well. It could be very important to establish any differences or similarities based on the serovar of the bacterium as each serovar of *C. trachomatis* is able to invade a different area of the human body and cause a different type of infection. For example, serovars A-C, which are known to cause trachoma and are not considered to be STDs, may need different regions of the Tarp protein or even may be able to invade host cells without Tarp while serovars L1-L3, which invade deep tissues and lymph nodes, may use a slightly different method of invasion [1]. If these knockouts prove to be successful, then it may also be advantageous to establish an infection model in animals such as chimpanzees or other monkeys that are similar to humans. It may also be possible to establish a model in mice but a different bacterium will have to be used. *C. trachomatis* infections are not optimal in mice and thus will not be guaranteed to give us any useful results. Instead, it may be possible to base a model around the use of *Chlamydia muridarium*, a mouse pathogen that has been used to study human *C. trahcomatis* infections in the past due to their similarities in how they cause genital tract infections [60]. Successful use of these sorts of models will allow us to further our research into seeing the effects that these knockouts may have on the symptoms of *C. trachomatis*.

One final area of interest that could prove useful for further investigation would be to extend research into seeing the effect that the Tarp molecule has within the entire *Chlamydia* genus and if it is also a necessary component in the process of invasion of host cells within *C. pneumoniae* and *C. psittaci*. This can be achieved in the same ways as observation of the different serovars and by using the shuttle vector to knock out either sections of Tarp or the entire Tarp molecule itself. It will be of particular interest to see if any differences arise in the necessity of Tarp or any of its regions in species of *Chlamydia* other than *C. trachomatis* due to the fact that *C. trachomatis* is the only form of *Chlamydia* to have the Phosphorylation domain. Comparisons between species will be key to elucidating why the phosphorylation domain was formed and why only *C. trachomatis* maintains it. Currently, these experiments will not be able to be performed due to the fact that the National Institutes of Health (NIH) have placed a ban on transforming antibiotic resistance genes into many other species of *Chlamydia* that can infect humans including *C. pneumoniae* and *C. psittaci*. If this ban were to ever be lifted, then these experiments may prove to be an interesting avenue of research. The answer to this question may also lie in a separate study comparing Tarp to the SipC protein within *Salmonella* bacteria. Much like L2 *C. trachomatis* Tarp, SipC is capable of both actin nucleation and bundling activity. These genetic knock outs and subsequent analysis may allow us to draw more parallels between the two molecules to further elucidate the exact mechanisms on how Tarp works. It may even be possible to use a shuttle vector to transform SipC in the place of Tarp to see if they can serve the same function in the invasion of a host cell *in vivo*.

This research has been able to give us perspective on not only how important the Tarp gene is to the invasion and overall life cycle of *C. trachomatis* but also provides foundational data for future Tarp research. This research is very exciting because it has provided the first look at a new way to analyze Tarp *in vivo*. Hopefully, through the use of this shuttle vector and replacement of genomic Tarp, the virulence factor can be more thoroughly analyzed and its importance in the process of invasion can be further

investigated. It is my hope that this research is the first step on a long road to the development of a *C. trachomatis* vaccine, and that this damaging infectious disease can be contained and possibly even eradicated.

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